Advances in Spermatozoal Phylogeny and Taxonomy

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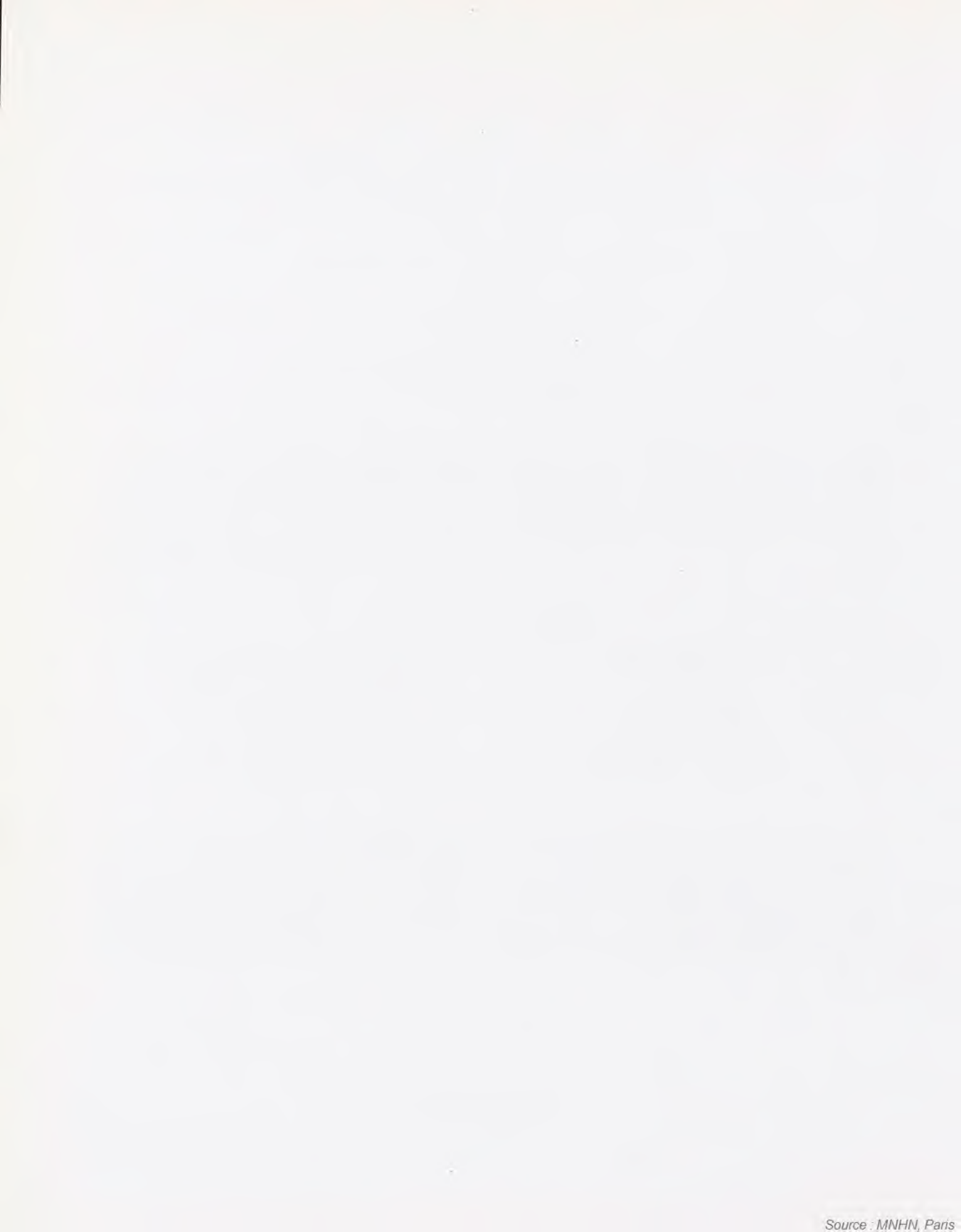

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Cover illustration:

The editors provided the artist, Pierre DIEGHI, with the illustrations of the book, with only two constraints: to observe the organisation of the book into three chapters, and to draw some cladograms in order to depict the phylogenetic spirit of the book. Complete freedom was otherwise given to him in the design of the illustrations.

Pierre is a bard of the Corsican culture. He has been seduced by the shape of some of the models, but makes it clear that it is the first time he has painted spermatozoa!

Illustration de couverture :

Les coordinateurs ont fourni à l'artiste un jeu des illustrations de ce volume, avec seulement deux contraintes : respecter l'organisation en trois chapitres, et figurer des cladogrammes pour indiquer l'orientation phylogénétique de l'ouvrage. Une totale liberté a été laissée au peintre pour le choix des illustrations, les dimensions et, bien sûr, les couleurs.

Pierre DIEGHI est peintre et chantre de la culture Corse. Il reconnaît avoir été séduit par la forme de certains des modèles, mais précise que c'est la première fois qu'il a peint des spermatozoïdes !

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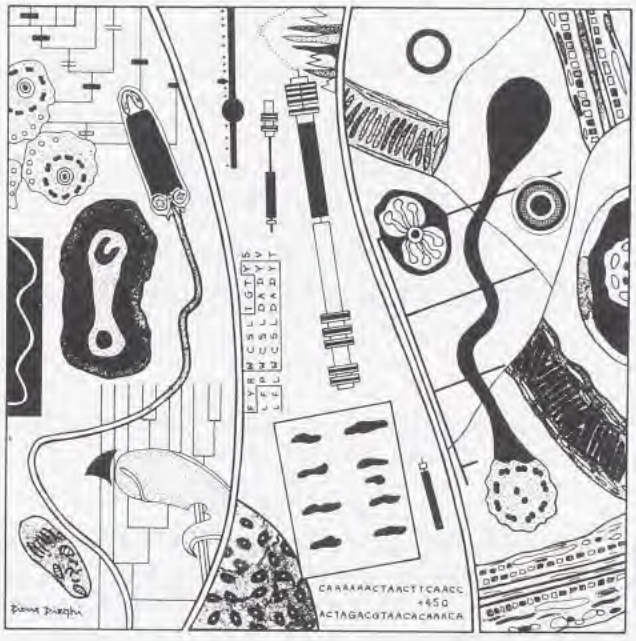
Advances in Spermatozoal Phylogeny and Taxonomy



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TOME 166
ZOOLOGIE

Advances in Spermatozoal Phylogeny and Taxonomy

edited by

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CONTENTS / SOMMAIRE

Pages

PREFACE 11

INVERTEBRATES AND GENERAL

1. **Outer arm dynein of sperm flagella and cilia in the animal kingdom** 15
Hideo MOHRI, Miyoko KUBO-IRIE & Masaru IRIE

2. **Amoeboid gametes and fertilization in *Dictyostelium*: gamete and pronuclear fusion are mediated by calmodulin and its binding proteins** 23
Danton H. O'DAY, Keith E. LEWIS & Michael A. LYDAN

3. **Sperm and spermiogenesis of the "Turbellaria" and implications for the phylogeny of the Phylum Platyhelminthes** 37
Nikki A. WATSON & Klaus ROHDE

4. **Spermatozoal ultrastructure and phylogeny in the parasitic Platyhelminthes** 55
Jean-Lou JUSTINE

5. **Spermiogenesis, spermatozoa and phyletic affinities in the Cestoda** 87
Cheikh Tidiane BÂ & Bernard MARCHAND

6. **Immunocytochemistry of tubulin in spermatozoa of Platyhelminthes** 97
Carlo IOMINI, Olga RAIKOVA, Nezha NOURY-SRAÏRI & Jean-Lou JUSTINE

7. **Comparative spermatology of Gastrotricha** 105
Marco FERRAGUTI & Maria BALSAMO

8. **Centrioles with ten singlets in spermatozoa of the parasitic nematode *Heligmosomoides polygyrus*** 119
Aïcha MANSIR & Jean-Lou JUSTINE

9. **Ultrastructure of sperm and sperm-egg interaction in Aculifera: implications for molluscan phylogeny** 129
John BUCKLAND-NICKS

10. **Comparative spermatozoal ultrastructure and its taxonomic and phylogenetic significance in the bivalve order Veneroida** 155
John M. HEALY

11. **Spermatozoal morphology of Patellogastropoda and Vetigastropoda (Mollusca: Prosobranchia)** 167
Alan N. HODGSON

12. **Comparative silver staining of molluscan spermatozoa** 179
Mário SOUSA, Elsa OLIVEIRA, João CARVALHEIRO & Víctor OLIVEIRA

13. **The use of spermatozoal ultrastructure in phylogenetic studies of Tubificidae (Oligochaeta)** 189
Christer ERSÉUS & Marco FERRAGUTI

14. **Comparative spermatology of Chelicerata: review and perspective** 203
Gerd ALBERTI

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15. **Spermatozoal ultrastructure in Dendrobranchiata (Crustacea, Decapoda): taxonomic and phylogenetic considerations** 231
Antonio MEDINA

16. **The atypical sperm morphologies of *Aristeus antennatus* and *Aristaeomorpha foliacea* (Crustacea, Dendrobranchiata, Aristeidae) and their phylogenetic significance** 243
Antonio MEDINA

17. **Ultrastructure and phylogeny of the spermatozoa of the infraorders Thalassinidea and Anomura (Decapoda, Crustacea)** 251
Christopher C. TUDGE

18. **Phylogeny of the Brachyura (Crustacea, Decapoda): evidence from spermatozoal ultrastructure** 265
Barrie G. M. JAMIESON, Danièle GUINOT & Bertrand RICHER DE FORGES

19. **Nuclear alterations during spermiogenesis of *Triatoma infestans* (Hemiptera, Reduviidae)** 285
Heidi DOLDER

20. **Sperm ultrastructure of *Xenos vesparum* (Rossi) and its significance in the taxonomy and phylogeny of Strepsiptera (Insecta)** 291
Marcella CARCUPINO, Giuseppe PROFILI, Jeyaraney KATHIRITHAMBY & Massimo MAZZINI

21. **Characteristics of the spermatozoon of *Cosmopolites sordidus* (Coleoptera: Curculionidae)** 297
José LINO NETO & Heidi DOLDER

22. **Phylogenetic significance of axonemal ultrastructure: examples from Diptera and Trichoptera** 301
Romano DALLAI & Björn A. AFZELIUS

VERTEBRATES

23. **Comparative morphology of the sperm in chondrichthyan fishes** 313
Sho TANAKA, Hana KUROKAWA & Masako HARA

24. **Comparative spermatology of anurans with special references to phylogeny** 321
Ae Sook KWON & Young Hwan LEE

25. **Amphibian sperm: phylogeny and fertilization environment** 333
Gerhard VAN DER HORST, Brian WILSON & Alan CHANNING

26. **Evolution of tetrapod spermatozoa with particular reference to amniotes** 343
Barrie G. M. JAMIESON

27. **The ultrastructure of spermatozoa of the Squamata (Reptilia) with phylogenetic considerations** 359
Barrie G. M. JAMIESON

28. **Ultrastructure of spermatozoa of australian blindsnakes, *Ramphotyphlops* spp. (Typhlopidae, Squamata): first observations on the mature spermatozoon of scolecophidian snakes** 385
H. Ronnie HARDING, Ken P. APLIN & Maria MAZUR

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29. **Ultrastructural and light microscopic observations of mature epididymal spermatozoa and sperm maturation of the Greater Bilby, *Macrotis lagotis* (Metatheria, Mammalia)** 397
Stephen JOHNSTON, Lina DADDOW & Franck CARRICK

30. **Variation in sperm head morphology of muroid rodents of Africa: phylogenetic implications** 409
William G. BREED

31. **Comparative sperm structure in bats (Chiroptera): some taxonomic and adaptive implications** 421
Takayuki MORI

32. **Molecular and ontogenic analysis of the human sperm tail fibrous sheath** 431
Ali JASSIM

33. **Diversity of avian spermatozoa ultrastructure with emphasis on the members of the order Passeriformes** 437
Lawrence D. KOEHLER

PROTEINS

34. **Histone H1 and the evolution of the nuclear sperm-specific proteins** 447
Juan AUSIO

35. **Evolution and origins of sperm nuclear basic proteins** 463
Harold E. KASINSKY

36. **Male germ line specific histones of sea urchins and sea stars** 475
Dominic POCCIA

37. **The gene encoding the sperm-specific basic nuclear protein ϕ_o from sea cucumber** 491
Eva PRATS & Luis CORNUDELLA

38. **Nuclear basic proteins from the sperm of tunicates, cephalochordates, agnathans and fish** 501
Manel CHIVA, Núria SAPERAS, Carme CÀCERES & Juan AUSIO

39. **Heparin-binding proteins on bull, boar, stallion, and human spermatozoa** 515
Juan José CALVETE, Libia SANZ, Markus REINERT, Zuzana DOSTÁLOVÁ & Edda TÖPFER-PETERSEN

40. **Histone gene expression during mammalian spermatogenesis: structural and functional aspects** 525
Detlef DOENECKE & Birgit DRABENT

41. **Sequence, evolution and transcriptional regulation of avian-mammalian P1 type protamines** 537
Rafael OLIVA

INDEX 549

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Preface / *Préface*

With the approach of the Seventh International Symposium on Spermatology at Cairns, Australia, in 1994, one of us (BGMJ) conceived the idea of editing a book which would cover recent advances in the use of spermatozoal ultrastructure in phylogeny and taxonomy. Contributions were invited not only from those attending the section of the Symposium in this field but also from non-participants. Such was the response to the invitations that the aid of a further person (JLJ) active in the field of spermiocladistics was sought in the exacting task of editing the contributions. The need for some treatment of advances in knowledge of sperm nuclear proteins led to the involvement of a third editor (JA) and raised the total number of chapters to 41, with 73 authors.

It was not to be expected that contributions on all animal groups would be received nor could they have been published in a single volume. Nevertheless, the authors have provided a most impressive testimony to the vigour of the burgeoning discipline of spermatozoon-based taxonomy and of the great utility of spermatozoal morphology and particularly ultrastructure as what has been termed an "independent arbiter" of conflicting hypotheses of phylogenetic relationship. The contributions also constitute a contemporary documentation of fine structure of the spermatozoon which will prove invaluable for students of reproductive biology. Several chapters deal also with the functional significance of components of the sperm cell.

The scope of the volume extends beyond the confines of the Animal Kingdom in a study of fertilization and calmodulin-mediated events in the slime mould *Dictyostelium*, valuable, inter alia, in exemplifying the simplest, amoeboid level of the sperm cell which may also have typified the earliest Metazoa. Variations in the molecular structure of dynein arms are demonstrated and shown to be specific to higher metazoan taxa.

In the invertebrate section, the value of sperm ultrastructure for phylogeny and taxonomy is demonstrated in a suite of chapters on the Platyhelminthes (providing synapomorphies for the majority of supra-generic groups in this phylum), in the most complete survey of chelicerate sperm to date, in reviews of thalassinidean, anomuran and brachyuran sperm and in those of dendrobranchiate shrimps; and in studies on bivalve and prosobranch mollusc sperm. In contrast, a parsimony analysis of tubificid sperm shows that although higher oligochaete and other clitellate groups are well defined spermatologically, high homoplasy largely masked relationships within the Tubificidae. The taxonomic significance of sperm structure in strepsipteran and hemipteran insects is also investigated and hitherto unknown taxonomic variation in axonemal structure in dipteran and trichopteran sperm is revealed. An elegant study of gastrotrich sperm shows profound differences between their two orders and a sperm morphology which is arguably the most bizarre known. An investigation of nematode sperm reveals the first centriole known to have 10 singlets. The remarkable similarity of the complex sperm of some aculiferan molluscs and protodrilid annelids is cited in support of relationships of their two

À l'approche du Septième Symposium International de Spermatologie à Cairns (Australie), l'un d'entre nous (BGMJ) conçut l'idée de coordonner un ouvrage qui couvrirait les apports récents de l'utilisation de l'ultrastructure des spermatozoïdes pour la phylogénie et la taxonomie. Des invitations à contribuer furent envoyées, non seulement aux participants du Symposium, mais aussi à d'autres chercheurs. Les réponses furent si nombreuses que l'aide d'une personne supplémentaire (JLJ) active dans le domaine de la spermatologie cladistique fut recherchée, en particulier pour la tâche exigeante de la vérification des contributions. La nécessité de la couverture des apports concernant les protéines nucléaires des spermatozoïdes amena au recrutement d'un troisième coordinateur (JA) et augmenta finalement le nombre d'articles à quarante et un, avec soixante-treize auteurs.

Nous n'avions jamais espéré que des contributions concernant tous les groupes animaux pussent être reçues ou même être réunies en un volume unique. Néanmoins, les auteurs ont fourni un témoignage impressionnant de la vigueur de cette discipline qu'est la taxonomie basée sur les spermatozoïdes, et de la grande utilité de la morphologie des spermatozoïdes, en particulier ultrastructurale, pour fournir ce qui a été appelé un "arbitrage indépendant" quand plusieurs hypothèses de relations phylogénétiques sont en conflit. Les contributions constituent aussi une documentation actualisée sur l'ultrastructure des spermatozoïdes, d'une valeur incomparable pour les chercheurs en biologie de la reproduction. Plusieurs chapitres concernent, de plus, la signification fonctionnelle des organites des spermatozoïdes.

Le sujet de ce volume s'étend au delà des confins du Règne Animal, dans une étude de la fécondation et des événements modulés par la calmoduline chez le Myxomycète Dictyostelium, *dont l'un des intérêts est de donner l'exemple du niveau le plus simple et amiboïde de spermatozoïde, qui pourrait aussi être typique des premiers Métazoaires. Une autre étude démontre que les variations de la structure moléculaire des bras de dynéine sont spécifiques des taxons supérieurs des Métazoaires.*

La section sur les Invertébrés démontre la valeur de l'ultrastructure des spermatozoïdes pour la phylogénie et la taxonomie: dans une série de chapitres concernant les Plathelminthes et fournissant des synapomorphies pour la majorité des groupes supra-génériques de cet embranchement; dans la révision publiée la plus complète concernant les spermatozoïdes des Chélicérates; dans les articles sur les spermatozoïdes de Crustacés incluant des synthèses sur les Thalassinides, les Anomoures et les Brachyoures, et un article sur les Dendrobranchiata; dans des études sur les spermatozoïdes des Mollusques Bivalves et Prosobranches. A l'opposé, une analyse de parcimonie sur les spermatozoïdes des Tubificidae montre que les homoplasies masquent les relations dans cette famille, bien que la spermatologie comparée permette de bien définir les grands groupes d'Oligochètes et les autres Clitellés. Chez les Insectes, des études concernent aussi la signification taxonomique de la structure des spermatozoïdes chez les Strepsiptères et les Hémiptères, et des variations inédites de l'ultrastructure des axonèmes sont décrites chez les Diptères et les Trichoptères. Une étude élégante des spermatozoïdes des Gastrotriches montre des différences profondes entre les deux ordres qui les constituent, et une morphologie du spermatozoïde que l'on peut considérer raisonnablement comme la plus bizarre jamais décrite. Une étude sur les spermatozoïdes des Nématodes révèle l'existence du premier centriole connu à dix singulets. Dans un autre article, la similarité remarquable entre les

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phyla, for which molecular evidence has recently emerged, and in favour of an hypothesis of complex introsperm as the primitive metazoan sperm.

As alternative techniques, silver staining of the acrosomal vesicle and cytoskeletal elements reveals species specific patterns of phylogenetic significance; and immunocytochemical studies of tubulin in platyhelminth sperm offer a new technique for elucidating sperm structure. Refined tannic acid techniques are provided for demonstrating details of axonemal structure.

In the vertebrate section, the remarkable diversity and great taxonomic and phylogenetic utility of sperm structure is demonstrated for chondrichthyan fish; for anurans, and other lissamphibians, in which sperm motility patterns are shown to be species specific and to relate to fertilization biology; for squamate reptiles, in which an hypothesis of relationship of eugongyloid, but not sphenomorph skinks, with snakes and pygopods merits consideration, and placement of typhlopods in the Serpentes is upheld; for amniotes as a whole, in which spermatozoal synapomorphies are found for most major taxa; for African murid rodents; for Chiroptera; and for passeriform birds. Spermatozoal ultrastructure of an endangered marsupial, the Greater Bilby, endorses the discreteness of its family Thalacomyidae. The function of proteins from the fibrous sheath of human sperm is discussed.

The third section, on sperm proteins, ranges widely over the evolution and nature of nuclear proteins throughout the Animal Kingdom; specific histones of the male germ line in echinoderms; heparin-binding proteins of mammalian sperm; genes encoding nuclear proteins; histone gene expression in mammalian spermatogenesis; and sequence, evolution and transcriptional regulation of avian -mammalian protamines.

The work of preparing a book by three editors separated by thousands of kilometres was not easy. After preliminary meetings and discussion in Cairns, progress was greatly aided by the generosity of the MNHN in hosting BGMJ as Invited Professor for three months during the summer of 95. JLJ carried out the work of preparing the photoready copy. The editors acknowledge an extensive use of the Internet between us, and with most authors, without which this book could not have been completed in reasonable time.

This volume is published in the series "Mémoires du Muséum" which is known for major contributions published in the field of systematics. Non-systematic plenary papers of the Proceedings of the Cairns meeting have been published by CSIRO in an independent volume.

We hope that this volume will set the stage for quantum advances by the next symposium on Spermatology, at Montreal in 1998.

spermatozoïdes des Mollusques Aculifères et des Annélides Protodrilides fournit un argument en faveur de relations phylogénétiques proches entre ces deux groupes, récemment démontrées par des preuves moléculaires, et de l'hypothèse de la présence d'un introspermatozoïde chez les Métazoaires primitifs.

Des techniques originales sont aussi décrites, telles que le marquage à l'argent des vésicules acrosomiennes et des éléments du cytosquelette, qui révèle des structures spécifiques d'intérêt phylogénétique, et l'immunocytochimie de la tubuline chez les Plathelminthes, qui permet de mieux comprendre la structure des spermatozoïdes. Les techniques raffinées de fixation à l'acide tannique permettent aussi de montrer des détails de la structure de l'axonème.

La section sur les Vertébrés décrit la diversité remarquable, et l'utilité indéniable, de la structure des spermatozoïdes pour la taxonomie et la phylogénie. Les études concernent: les Chondrichtiens; les Anoures et les Lissamphibiens, chez lesquels il est montré que les types de motilité sont spécifiques et en rapport avec la biologie de la fécondation; les Reptiles Squamata, pour lesquels une hypothèse de relations des Eugongyloïdes, mais non des Scincidae Sphénomorphes, avec les Serpents et les Pygopodes mérite considération, et pour lesquels on maintient l'inclusion des Typhlopodes dans les Serpents; les Amniotes dans leurs ensemble, chez lesquels des synapomorphies du spermatozoïde existent pour la plupart des taxons majeurs; les Rongeurs Muridae africains; les Chiroptères, et les Oiseaux Passeriformes. L'ultrastructure du spermatozoïde chez une espèce menacée, le Grand Lièvre marsupial, confirme le statut particulier de la famille Thalocomyidae à laquelle elle appartient. La fonction des protéines de la gaine fibreuse des spermatozoïdes humains est discutée.

La troisième section, sur les protéines des spermatozoïdes, concerne de manière très large l'évolution et la nature des protéines nucléaires dans l'ensemble du Règne Animal, et aussi: les histones spécifiques de la lignée germinale mâle chez les Échinodermes; les protéines se liant à l'héparine dans les spermatozoïdes des Mammifères; les gènes codant pour les protéines nucléaires; l'expression des gènes des histones pendant la spermatogenèse des Mammifères; et la séquence, l'évolution et la régulation transcriptionnelle des protamines des Oiseaux et Mammifères.

Il n'est pas facile de préparer un livre quand les trois coordinateurs sont séparés par des milliers de kilomètres. Après des rencontres préliminaires à Cairns, cette tâche a pu être menée à bien grâce à la générosité du MNHN qui a accueilli BGMJ comme Professeur Associé pendant les trois mois de l'été 1995. JLJ a effectué la mise en page en PAO. Les coordinateurs reconnaissent l'aide apportée par l'utilisation intensive d'Internet, entre eux, et avec la plupart des auteurs, aide sans laquelle cet ouvrage n'aurait pas pu être terminé dans un temps raisonnable.

Cet ouvrage est publié dans la collection des "Mémoires du Muséum", bien connue pour ses contributions majeures dans le domaine de la Systématique. Les conférences plénières du Symposium de Cairns ne relevant pas de la Systématique sont publiées par le CSIRO dans un volume indépendant.

Nous espérons que ce volume ouvrira la voie à des progrès significatifs lors du prochain symposium de Spermatologie de 1998, à Montréal.

Barrie JAMIESON (Brisbane)
Juan AUSIO (Victoria)
Jean-Lou JUSTINE (Paris)

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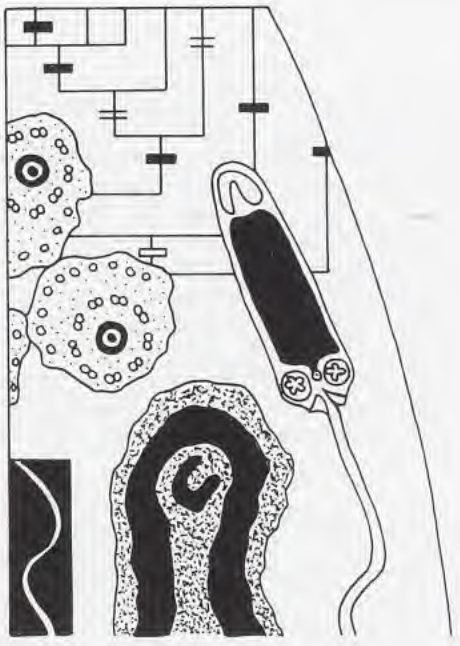
Invertebrates and General



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Outer Arm Dynein of Sperm Flagella and Cilia in the Animal Kingdom

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ABSTRACT

It has been established that the outer arm dynein molecule of flagella and cilia in Protozoa such as *Chlamydomonas* and *Tetrahymena* has a three-headed structure, whereas that of sperm flagella in sea urchins, fish and mammals exhibits a two-headed structure. All the latter animals belong to Deuterostomia. The outer arm dynein from sperm flagella of the oyster, a member of Protostomia, is also two-headed. Investigation has begun of the situation in other animals, especially in Cnidaria and Porifera. In *Chlamydomonas*, there is a mutant whose outer arm dynein lacks one head. Electron microscopy revealed that the outer arm of the wild type shows a pistol-like shape in the cross-section of the flagellar axoneme, whereas that of the mutant exhibits a hook- or fist-like shape. This suggests that we may predict the number of heads of outer arm dynein by observing the cross-sections of flagella or cilia. Examination of the cross-sections of sperm flagella and cilia in various animals, including mammals, tunicates, echinoderms, molluscs, annelids, arthropods, flatworms and sea anemones, indicated that their outer arms were hook- or fist-like, in contrast to the pistol-like shape in *Paramecium* and *Tetrahymena*. The former was true with choanocyte flagella of the sponge and metazoan cilia. The reduction in the number of heads of outer arm dynein molecule would have occurred during the evolution from Protozoa to Metazoa (and Mesozoa). Alternatively, the outer arm dynein in Protozoans was specialized to support their peculiar behaviour.

RÉSUMÉ

La dynéine du bras externe des flagelles des spermatozoïdes et des cils dans le Règne Animal

Il a été établi que la molécule de dynéine du bras externe des flagelles et des cils des Protozoaires, tels que *Chlamydomonas* et *Tetrahymena*, a une structure à trois têtes, alors que les flagelles des spermatozoïdes des Oursins, Poissons et Mammifères ont une structure à deux têtes. Tous les animaux précédemment cités appartiennent aux Deutérostomiens. La dynéine du bras externe de l'huître, un Protostomien, a aussi deux têtes. Les études sur d'autres animaux, spécialement des Cnidaires et des Porifères, ont commencé. Chez *Chlamydomonas*, il existe un mutant dont la dynéine du bras externe est dépourvue d'une des têtes. La microscopie électronique montre que le bras externe de type sauvage a une forme en pistolet sur les coupes transversales de l'axonème du flagelle, alors que celui du mutant a une forme en crochet ou en poing. Ceci suggère que nous pouvons prédire le nombre de têtes de la dynéine du bras externe en observant des coupes transversales de flagelles ou de cils. L'observation de coupes transversales de flagelles de spermatozoïdes et de cils d'animaux divers, tels que des Mammifères, Tuniciers, Échinodermes, Mollusques, Annélides, Arthropodes, Plathelminthes et Anémones de Mer, montre que leur bras externe est en forme de crochet ou de poing, en opposition avec la forme en pistolet de *Paramecium* et *Tetrahymena*. La même situation a été rencontrée pour les flagelles des choanocytes des Spongiaires et les cils des Métazoaires. La réduction du nombre de têtes de la molécule de dynéine du bras externe a pu intervenir lors de l'évolution depuis les Protozoaires vers les Métazoaires (et Mésozoaires). Une alternative serait d'interpréter la dynéine du bras externe des Protozoaires comme spécialisée pour permettre leur comportement particulier.

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Source : MNHN, Paris

Dynein was first thought to be a globular molecule with molecular mass of 300 000-600 000, representing either a single outer arm or an inner arm in the axoneme of flagella and cilia [7, 16]. The outer arm dynein has three heads connected by stalks to a common base in flagella or cilia of Protozoa such as *Chlamydomonas* [8, 26], *Tetrahymena* [11, 28] and *Paramecium* [13], as revealed by either negatively by stained or quick-freeze deep-etch images under the electron microscope. Biochemical analyses also indicated that there are three distinct heavy chains with molecular masses of 400 000 - 500 000, which constitute the corresponding heads, as well as a few intermediate chains and a few light chains. On the other hand, outer arm dynein obtained from sperm flagella of sea urchin [24], rainbow trout [6] and bull [14] has two heads and consists of the corresponding two heavy chains. Recent studies indicate that inner arm dynein is different from outer arm dynein not only in molecular composition [19] but also in function [4, 5]. A couple of different inner arm dynein molecules are present in a single flagellum and cilium, and they appear to be two-headed even in the case where outer arm dynein is three-headed [21, 25]. Phylogenetically, all the above-mentioned Metazoa belong to Deuterostomia.

We examined the outer arm dynein of sperm flagella in the oyster as a representative of Protostomia [30]. The purified dynein contained two heavy chains, and its negative stained image was two-headed under the electron microscope. However, the question arose of whether the two-headed dynein obtained is really an intact molecule or the product of a three-headed molecule as 18S dynein in *Chlamydomonas* is [33], although the same extraction procedure was used as that applied for outer arm dynein from sea urchin sperm flagella. It is desirable to check this point by some other means. Furthermore, from a phylogenetic point of view, it is interesting to know whether the outer arm dynein of flagella or cilia in other animals, especially Cnidaria, Porifera, etc., is two-headed or three-headed.

TABLE 1. — List of species examined. (s): sperm, (f): flagella, (c): cilia.

Protozoa	*Chlamydomonas reinhardtii* (f)
	Tetrahymena pyriformis (c)
	Paramecium caudatum (c)
Porifera	*Halichondria japonica* (f)
Cnidaria	*Anthopleura midori* (s)
Platyhelminthes	*Planoceros reticulata* (s)
Annelida	*Neanthes diversicolor* (s)
Mollusca	*Crassostrea gigas* (s, c)
	Mytilus edulis (s)
	Meretrix lusoria (c)
	Tapes japonica (c)
Arthropoda	*Balanus uliginosus* (s)
	Callophrys ferrea (s) and other butterflies (s)
Echinodermata	*Hemicentrotus pulcherrimus* (s)
	Pseudocentrotus depressus (s)
	Anthocidaris crassispina (s)
Protochordata	*Ascidia sydneiensis semea* (s)
Vertebrata	*Mus musculus* (s, c)

In *Chlamydomonas*, there is a flagellar mutant (oda-11) missing the α- heavy chain of outer arm dynein but retaining the β- and γ-chains [22]. In other words, the outer arm dynein molecule in this mutant is two-headed. Examination of the cross-sections of axonemes under the electron microscope indicates that the image of the outer arm in wild-type *Chlamydomonas* is pistol-like

Source : MNHN, Paris

(see Fig. 1a), whereas that in the mutant is hook- or fist-like (see Fig. 1b). This fact should provide us a useful tool for predicting the number of heads of outer arm dynein molecules *in situ*. In fact, the image of the outer arm, consisting of two-headed dynein, in the cross-section electron micrograph of the flagellar axoneme of sea urchin sperm resembles that of the flagellar axoneme of the mutant *Chlamydomonas* (see Fig. 1c). Since the starting materials are often insufficient in amount for biochemical analysis of a purified dynein molecule, such an examination of the image of the outer arm on the cross-sections of flagella or cilia seem to be effective in comparatively analyzing the number of heads of the outer arm dynein molecule in different animal species. In the present study, the outer arms of sperm flagella or cilia were examined in the species listed in Table 1. In the case of Porifera, choanocyte flagella were used as material instead of sperm flagella.

MATERIALS AND METHODS

Flagellar axonemes of the wild type and oda 11 mutant of *Chlamydomonas reinhardtii* isolated by the method of WITMAN [32] and ciliary axonemes of *Tetrahymena pyriformis* isolated by the method of TANAKA & MIKI-NOUMURA [27] were gifts from Dr. R. KAMIYA and Dr. T. MIKI-NOUMURA, respectively. Sperm flagella and axonemes of the sea urchins *Hemicentrotus pulcherrimus*, *Psudocentrotus depressus* and *Anthocidaris crassispina*, of the molluscs *Crassostrea gigas* and *Mytilus edulis*, and of the sea anemone *Anthopleura midori*, were prepared as previously described [10]. Spermatozoa of the mouse *Mus musculus*, of the tunicate *Ascidia sydneiensis semea*, of the cirripede *Balanus uliginosus*, of the annelid *Neanthes diversicolor*, and of the flatworm *Planoceros reticulata* were directly treated with a demembranation solution consisting of 0.1-0.4 % Triton X-100, 0.15 M KCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM DTT and 20 mM Tris-HCl, pH 8.0. Intact *Paramecium caudatum*, a piece of mouse trachea and pieces of the gill of bivalves, *Crassostrea gigas*, *Meretrix lusoria* and *Tapes japonica*, were subjected to the demembranation solution. The sponge *Halichondria japonica* was minced with scissors, filtered through a mesh to remove spicules and then treated with the demembranation solution.

Demembranated axonemes of flagella and cilia (intact flagella in the cases of insect sperm) were fixed with a mixture of 2.5 % glutaraldehyde and 1 % tannic acid in 0.2 M sodium cacodylate, followed by either post-fixation with 1 % OsO_4 or block-staining with uranyl acetate. Specimens were dehydrated and embedded in Quetol 812 (Epon 812 in the case of *Chlamydomonas* flagella). Thin sections were made with a Sorvall ultra-microtome MT-2 and observed under a JEOL 1200A electron microscope. To examine the shape of the outer arms more thoroughly, images of the axonemes on cross-section electron micrographs were superimposed and averaged using an IBAS image analyzer.

RESULTS

Protozoan flagella and cilia

As mentioned in the Introduction, the outer arm of the wild-type *Chlamydomonas* flagella had a pistol-like shape on the cross-section electron micrograph (Fig. 1a). The image corresponds to a three-headed dynein molecule. On the other hand, that of the mutant (oda 11) flagella, in which the α-heavy chain of outer arm dynein molecule is missing, showed a hook- or fist-like image (Fig. 1b). Comparison of the averaged image of the outer arms between the wild type and the mutant clearly indicated that the distal portion of the outer arm is lost in the mutant flagella, as described by SAKAKIBARA *et al.* [22]. One head corresponding to the α-heavy chain must reside in this domain of the outer arm. As is shown in Fig. 2a, the image of the outer arm on the cross-section of *Tetrahymena* cilia was quite similar to that in the wild *Chlamydomonas* flagella. The same was true of *Paramecium* cilia (Fig. 2b). The results are consistent with the fact that isolated outer arm dynein molecules in these organisms are three-headed.

Metazoan flagella

Figure 1c shows the cross-section of the sea urchin sperm flagellum (*Hemicentrotus*) and the averaged image of the outer arm. It is clear that the image is hook- or fist-like, as in the case of the outer arm in the mutant *Chlamydomonas* flagellum. Figures 2c and 2e show the cross-sections of sperm flagella in the mouse and the oyster *Crassostrea*, whose outer arm dynein must be two-headed. In both cases the image of the outer arm was similar to that in the mutant *Chlamydomonas*. Thus the two-headed structure of outer arm dynein must be reflected in an image of the outer arm. The outer arm of sperm flagella in the tunicate *Ascidia*, a member of Deuterostomia as mammal and sea urchin, was also the two-headed type (Fig. 2d).

Source : MNHN, Paris

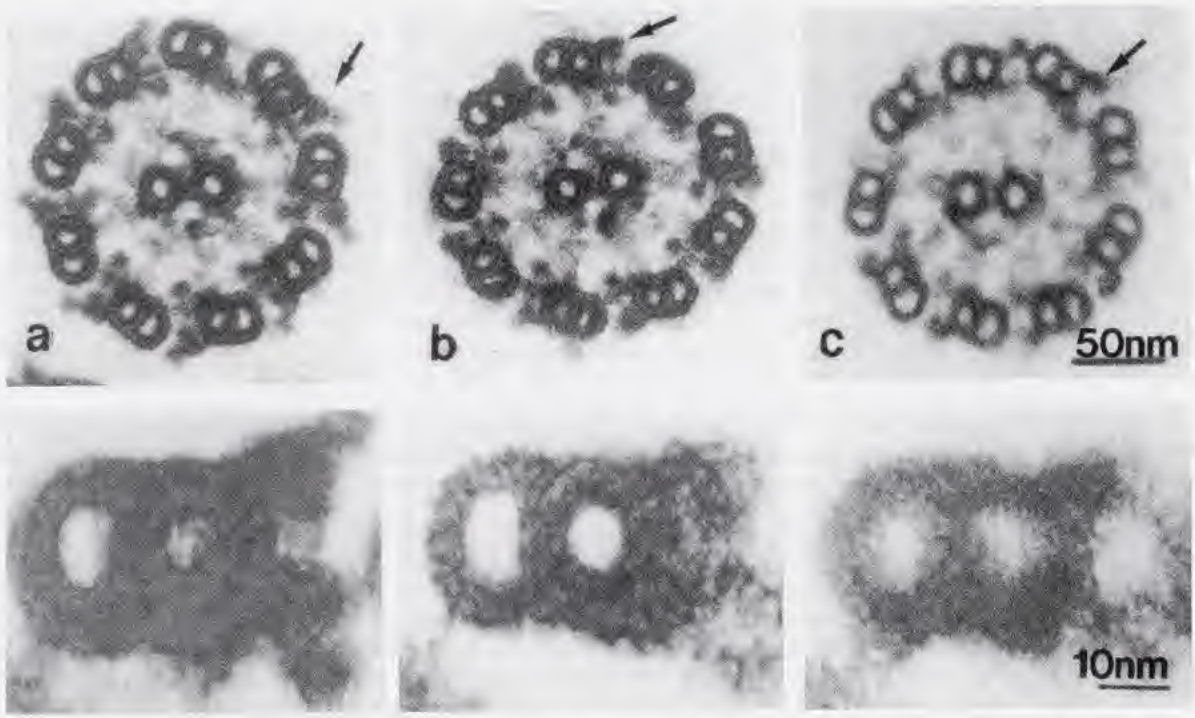

FIG. 1. — Cross-sections of axonemes with averaged outer doublet images. **a:** Flagellum of wild-type *Chlamydomonas*; **b:** Flagellum of a mutant (oda 11) *Chlamydomonas*; **c:** Sperm flagellum of the sea urchin *Hemicentrotus pulcherrimus*.

The results obtained with sperm flagella of other members of Protostomia are shown in Fig. 2g, h, i and j. They were the annelid *Neanthes*, the insect *Callophrys*, the cirripede *Balanus* and the flatworm *Planoceros*. The images of the outer arms in these species were also hook- or fist-like, indicating the two-headed molecule of outer arm dynein. Although not shown here, sperm flagella of many butterflies other than *Callophrys* were examined with the same result. In arthropods such as insects and cirripedes, the morphology of the outer arm was somewhat different from that in other animals, taking rather an axe-like shape (see also [1]). However, the image corresponding to the distal domain of the protozoan outer arm could not be found. It should be noted that the axoneme of flatworm sperm is a 9+"1" type.

Cnidaria is likely to be the phylogenetic ancestor of both Protostomia and Deuterostomia. A cross-section of sperm flagellum of the sea anemone, *Anthopleura*, is shown in Fig. 2k. The shape of the outer arm in this species was not different from that of sperm flagella in other metazoan animals. A preliminary biochemical analysis of purified outer arm dynein preparation in the sea anemone indicated the presence of two heavy chains on SDS polyacrylamide gel (data not shown). Furthermore, the outer arm of choanocyte flagella in the sponge *Halichondria* was not of the protozoan type (Fig. 2l).

Metazoan cilia

Since cilia were examined in *Tetrahymena* and *Paramecium*, and furthermore *Chlamydomonas* flagella beat like metazoan cilia in forward movement, metazoan cilia may possess outer arm dynein somewhat different from that of metazoan flagella. As can be seen from

Source : MNHN, Paris

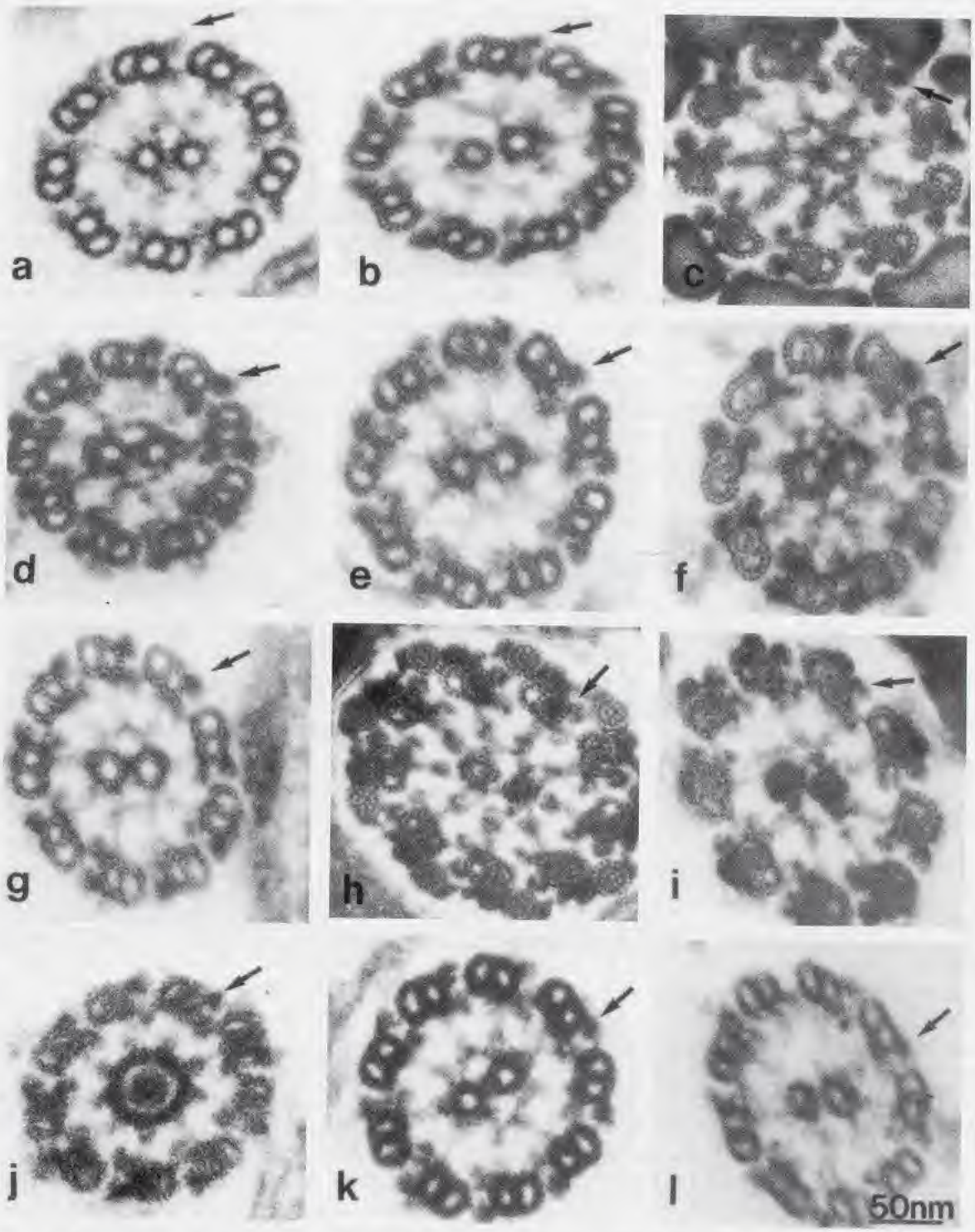

FIG. 2. — Cross-sections of flagellar and ciliary axonemes in various animals. **a:** Cilium of *Tetrahymena pyriformis*; **b:** Cilium of *Paramecium caudatum*; **c:** Sperm flagellum of the mouse *Mus musculus*; **d:** Sperm flagellum of the tunicate *Ascidia sydneiensis semea*; **e:** Sperm flagellum of the oyster *Crassostrea gigas*; **f:** Gill cilium of the oyster; **g:** Sperm flagellum of the annelid *Neanthes diversicolor*; **h:** Sperm flagellum of the butterfly *Callophrys ferrea*; **i:** Sperm flagellum of the cirripede *Balanus uliginosus*; **j:** Sperm flagellum of the flatworm *Planoceros reticulata*; **k:** Sperm flagellum of the sea anemone *Anthopleura midori*; **l:** Choanocyte flagellum of the sponge *Halichondria japonica*. Arrows indicate the outer arms.

Source : MNHN, Paris

Fig. 2f, the outer arm of gill cilia in the oyster shows the same image as that of sperm flagella of the same species. The results were the same with gill cilia of other bivalves, *Meretrix* and *Tapes*, and with tracheal cilia of the mouse.

DISCUSSION

Morphology of the outer arm

The presence of several dynein species with somewhat different functions has been disclosed within a single flagellum or cilium [20, 29]. According to a recent review by BROKAW [4], the main function of outer arm dynein in flagella and cilia appears to be generation of power to overcome viscous resistance, in contrast to the functions of inner arm dyneins, which appear to be bend initiation and maintenance of the angle of propagating bend. Outer arm dynein molecules so far purified from protozoan flagella and cilia possess three heads corresponding to three different heavy chains, α, β and γ, obtained by SDS-PAGE [8, 11, 13, 26, 28]. On the other hand, purified outer arm dynein molecules of sperm flagella in Metazoa such as mammals, fish, sea urchins and molluscs, consist of only two heads and two heavy chains, α and β [10, 13, 24, 30].

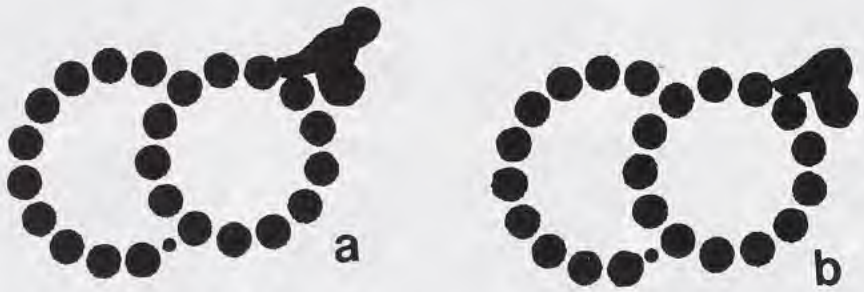

FIG. 3. — Schematic cross-section of an outer doublet microtubule with the outer arm. **a:** protozoan axoneme; **b:** metazoan axoneme.

Recent studies on *Chlamydomonas* flagellar mutants by KAMIYA and his colleagues [22, 23] clearly showed that one head corresponding to the α-heavy chain is situated at the distal domain of the outer arm, which looks like a pistol on the axoneme cross-section, the other two heads compose the body domain of the arm, one head corresponding to the γ-heavy chain localized at the innermost domain. Only the body domain of the outer arm is found in a flagellar mutant, oda 11, of *Chlamydomonas*, which lacks the α-heavy chain, resulting in a hook-like image of the outer arm on the cross-section electron micrograph. The present results indicate that in the metazoan sperm flagella from which two-headed outer arm dynein molecules have been isolated, the outer arms show the hook-like image representing the body domain of the outer arm in protozoan axonemes. In other words, α- and β-heavy chains of metazoan outer arm dynein would correspond to β- and γ-heavy chains of protozoan outer arm dynein. This fact also proves that the outer arm dynein molecule of oyster sperm flagella is certainly two-headed *in situ*, as indicated by biochemical analysis and the negative stained image of isolated dynein [30].

Most schematic cross-sections of flagellar and ciliary axonemes are based on electron micrographs obtained with either *Tetrahymena* cilia [2] or molluscan gill cilia [31], and have been accepted as common to all kinds of flagella and cilia [e.g: 3, 15]. Little attention has been paid to the image of the outer arm, in spite of the above-mentioned difference in the number of heads of

Source : MNHN, Paris

outer arm dynein. In the most recent diagram depicting the *Chlamydomonas* axoneme, the outer arm is drawn as it is in Fig. 1a in this paper ([12]; see also computer-generated cross-section of the axoneme in [9]). Therefore, a different image should be drawn for the metazoan axoneme. On the basis of the present findings, we propose here an interpretative diagram of the outer arms in protozoan and metazoan axonemes as represented in Fig. 3.

Phylogenetic aspects

Phylogenetically, sperm flagella of metazoan animals belonging to Protostomia and Deuterostomia exhibit the same morphology of the outer arm, which incorporates the two-headed structure of outer arm dynein. The outer arm of flatworm sperm flagella which have a 9+"1" axoneme and show three-dimensional movement is also not exceptional. No difference is observed with ciliary axonemes of these metazoan species, and outer arm dynein from embryonic cilia of the sea urchin has been reported to contain only two heavy chains [18]. Thus motility pattern does not seem to reflect on the structure of outer arm dynein among Metazoa.

An axe-like image of the outer arm in the cross-section electron micrographs of arthropod sperm flagella (Fig. 2h, i) would be caused by a slight shift of the two heads inward as compared with the arrangement of these heads in other metazoan axonemes. Down the phylogenetic tree to Cnidaria and Porifera, the common ancestors of both Protostomia and Deuterostomia, outer arm dynein molecules are considered to be two-headed. Furthermore, the image of the outer arm in the cross-section of the ciliary axoneme of Mesozoa, *Dicyema misakiensis*, is also hook- or fist-like, suggesting the two-headed structure of constituent dynein (Y. YAMAKAWA & M. OKUNO, personal communication). Although more thorough examination would be needed, so far only protozoan flagella and cilia have outer arm dynein with a three-headed structure.

Hence the reduction in the number of heads of outer arm dynein molecule would have occurred during evolution from Protozoa to Mesozoa and Metazoa, as an adaptation to simplify and make more efficient the motile machinery of the latter's flagella and cilia. Alternatively, the outer arm dynein in protozoan flagella and cilia was rather specialized. The addition of an extra head would facilitate complex movements such as the cilia-like motion in forward swimming and flagella-like motion in backward swimming of *Chlamydomonas* as each of the heads of outer arm dynein appears to exhibit a somewhat different function from the others [17].

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Source : MNHN, Paris

Amoeboid Gametes and Fertilization in *Dictyostelium:* Gamete and Pronuclear Fusion are Mediated by Calmodulin and its Binding Proteins

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ABSTRACT

The gametes of *Dictyostelium discoideum* are tiny amoeboid cells which contain a small condensed nucleus. Gamete formation is calcium-independent and inhibited by calmodulin function. Accumulated data suggests that the gametes are a source of sexual pheromone that promotes sexual cell fusion. Time-lapse videomicroscopy has revealed that gametes are highly motile compared to non-gametic cells and, when two gametes contact, fusion results in the formation of a binucleate cell. Within each binucleate cell, pronuclear migration, swelling and fusion occur as the cytoplasmic volume of the cell increases dramatically producing a zygote giant cell. Gamete fusion is calcium-dependent and involves at least one membrane-bound, GlycNAc-containing glycoprotein (gp138). Fertilization is mediated by the dual signal transduction pathway involving calcium and protein kinase C. Of particular importance is the downstream role of calmodulin (CaM) and its binding proteins (CaMBPs). A putative CaM Kinase III activity and two, as yet unidentified CaMBPs (i.e. CaMBP-48, CAMBP-91) are developmentally regulated and temporally associated with the events of cell and pronuclear fusion in *D. discoideum*. Fertilization is terminated by a feed-back mechanism involving an autoinhibitor that is secreted by the zygote giant cells. This low molecular weight, hydrophobic, heat-stable autoinhibitor inhibits both cell and pronuclear fusion by preventing the interaction of CaM with its binding proteins. These results are discussed in terms of fertilization and signal transduction involving calmodulin and its binding proteins in higher animals.

RÉSUMÉ

Gamètes amiboïdes et fécondation chez *Dictyostelium:* la calmoduline et ses protéines liées sont les médiateurs de la fusion des gamètes et des pronucleus

Les gamètes de *Dictyostelium discoideum* sont des petites cellules amiboïdes qui contiennent un petit noyau condensé. La formation des gamètes est indépendante du Calcium et est inhibée par un système à calmoduline. Une abondance de données suggère que les gamètes sont une source de phéromone sexuelle qui déclenche la fusion sexuelle des gamètes. La vidéomicroscopie a montré que les gamètes sont hautement mobiles en comparaison avec les cellules non gamètes, et que, quand deux cellules entrent en contact, la fusion a pour résultat la formation d'une cellule binucléée. Dans chaque cellule binucléée, la migration, le gonflement et la fusion des pronucléus interviennent alors que le volume cytoplasmique de la cellule augmente de manière importante en produisant une cellule zygote géante. La fusion des gamètes est dépendante du Calcium et implique au moins une glycoprotéine liée à la membrane contenant GlycNac (gp138). La fécondation fait intervenir le système de transduction double impliquant le Calcium et la protéine kinase C. Le rôle en aval de la

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O'DAY, D. A. H., LEWIS, K. E., & LYDAN, M. A., 1995. — Amoeboid gametes and fertilization in *Dictyostelium:* gamete and pronuclear fusion are mediated by calmodulin and its binding proteins. *In*: JAMIESON, B. G. M., AUSIO, J., & JUSTINE, J.-L. (eds), Advances in Spermatozoal Phylogeny and Taxonomy. *Mém. Mus. natn. Hist. nat.*, **166** : 23-36. Paris ISBN : 2-85653-225-X.

Source : MNHN, Paris

calmoduline (CaM) et de ses protéines liées (CaMBPs) est d'une importance particulière. Une activité CaM Kinase III supposée et deux CaMBPs jusqu'ici non identifiées (CaMBP-48 et CaMBP-91) sont régulées au cours du développement et associées temporellement avec les événements de fusion des cellules et des pronucléus chez *D. discoideum*. La fécondation est achevée par un mécanisme en retour impliquant un autoinhibiteur qui est sécrété par les cellules zygotes géantes; cet autoinhibiteur, qui est de petit poids moléculaire, hydrophobe, stable à la température inhibe à la fois la fusion des cellules et des pronucléus en empêchant l'interaction de la CaM avec ses protéines liées. Ces résultats sont discutés en comparaison avec la fécondation et la transduction de signal impliquant la calmoduline et ses protéines liées dans les animaux supérieurs.

The importance of signal transduction during fertilization and other developmental processes has only recently come to light. In evolutionary terms, gamete fusion undoubtedly represents the first regulated type of cellular fusion wherein the cell membranes of two different cell types contact, bind and amalgamate to form a new cell type of unique genetic composition (i.e., a zygote). How do cells that have contacted relay their compatibility? How do they set in motion the sequence of events that will lead to their coalescence? These simple questions lie at the heart of fertilization of all organisms from simple eukaryotic microbes such as *Dictyoselium* to mammals such as mouse and humans.

While the probing of the sex life of *Dictyostelium* has only occurred during the last twenty years or so, research is facilitated by the extensive literature that exists and the powerful cellular, genetic and molecular methodologies that have been developed during studies on asexual development of this social amoeba. For these reasons, advances in understanding fertilization and sexual development in this model organism should progress rapidly in the future. Fertilization in *Dictyostelium discoideum* is comparatively simple (Fig. 1). Tiny, highly motile, amoeboid gametes appear when cells are cultured in the dark [57]. When two compatible gametes make contact they fuse to produce a binucleate cell. Pronuclear swelling, migration and fusion occur concomitantly with a large increase in cytoplasmic volume producing a zygote giant cell [43, 70]. The zygote giant cells takes over control of subsequent development in sexual cultures using several secreted molecules to regulate the behaviour of non-zygotic cells in the culture [1, 13, 24, 52, 64, 68]. In addition to inhibiting continued gamete cell fusion, this is reflected by each giant cell chemoattracting hundreds of other cells and then ingesting them in an act of cannibalistic phagocytosis [29, 30, 31, 32, 52].

As will be detailed in the work presented here, the events of fertilization in *Dictyostelium* are dependent on the functioning of calmodulin (CaM) and its binding proteins (CaMBPs). Calmodulin is present in the sperm and eggs of all animal species that have been studied and its function is essential to many of the component events of fertilization. For example, calmodulin mediates such diverse sperm functions as activation, motility, capacitation, the acrosome reaction as well as sperm-egg fusion (Table 1). As in other systems, calmodulin carries out its functions by regulating the activity of other proteins, often enzymes. A number of these calmodulin binding proteins have been isolated from spermatozoa from various species and in some cases specific roles have been attributed to them (Table 1). To date, comparatively little work has been done on calmodulin and its binding proteins in amoeboid spermatozoa.

The fusion of amoeboid gametes represents one of the simplest forms of eukaryotic fertilization. In spite of this, the fundamental events of species-specific cellular recognition, adhesion and cell fusion followed by pronuclear fusion that are common to all types of fertilization also occur [e.g. 79]. On the other hand, the absence of complex surface structures (e.g. egg coats) and other accessories (e.g. acrosome, acrosomal process, etc.) likely leaves us with a stripped-down system made up solely of the essential elements that are fundamental to regulated cell coalescence in all organisms. With this in mind, the aim of this article is to examine the state of knowledge of gamete formation and fertilization in *D. discoideum* and other slime mould species while keeping a general, comparative eye on some related work that has been done on fertilization in animals.

Source : MNHN, Paris

TABLE 1. — Localizations and roles of calmodulin and calmodulin binding proteins in various spermatozoa [1]

I. CALMODULIN

Organisms	Localization[2]	Function	Reference
Caenorhabditis elegans	unknown	inhibits onset of spermatogenesis	[66]
Sea urchin	unknown	acrosome reaction	[10, 18]
Boar, guinea pig	acrosome	acrosome reaction	[11, 17]
Rat, boar, guinea pig, sea urchin, man	flagellum	flagellar motility	[2, 17, 20, 49, 72]
Rabbit, hamster, mouse, rat, man, boar, monkey, guinea,pig	post-acrosomal sheath	sperm-egg fusion	[2, 9, 11, 20, 21, 72]

II. CALMODULIN BINDING PROTEINS

Organisms	Type of CaMBP	Function (Localization)	Reference
Thyone (Echinoderm)	Spectrin-like (profilactin cup)	formation of acrosomal process	[14]
Abalone	CaM-dependent adenylate cyclase	unknown	[23]
Sea urchin, boar	CaM-dependent adenylate cyclase	unknown	[77]
Sea urchin, dog, mouse, pig	CaM-dependent protein phosphatase calcineurin-like	flagellar motility (flagellum)	[20, 71]
Mouse	CaM-dependent protein phosphatase isoform type 3	flagellar motility	[72]
Hamster	Unknown CaMBP	sperm function (sperm head)	[47]
Rat, pig, bull	Calspermin, 32kDa 28, 30, 49 kDa CaMBPs	unknown negative regulation of capacitation	[78] [25]
Human	several 22-27 kDa and 32 kDa CaMBPs 67 kDa CaMBP	unknown unknown (flagellum)	[2] [61]

[1] This is a summary of selected references and is not meant to be a complete or comprehensive review of the literature or of all proposed functions of calmodulin and its binding proteins.

[2] The location of these molecules can vary with developmental stage, physiological state or experimental treatment.

Source : MNHN, Paris

RESULTS AND DISCUSSION

Gamete development

After mixed mating type strains (NC4 x V12) of *Dictyostelium discoideum* are grown together in the dark, a new tiny amoeboid cell type appears about 6 hours after the culture is started (Fig. 1) [57]. These cells have 1/4 of the cytoplasmic volume and 1/5 the nuclear volume of typical vegetative amoebas. Under phase contrast microscopy the cells are more birefringent than vegetative amoebae (Fig. 2a-c). When stained with Hoechst 33258, a fluorescent nuclear stain, these cells are seen to possess tiny nuclei that fluoresce brightly (Fig. 2d, e). Time-lapse videomicrography has shown that these tiny amoebae move much more quickly than vegetative cells. In their movements they make contacts with other cells, pause, then move on. Upon contact with another small amoeba, two cells of opposite mating type can fuse (Fig. 2f). In a series of elegant experiments, URISHIHARA & YANAGISAWA [73, 74] used cell ghosts to show that fusion occured between cells of opposite mating type. In cultures containing 1 mM calcium chloride, fusion is inititated around 8 hours after cultures are started. Fusion is initiated after contact of extended filopodia (Fig. 2f). The fusion of these tiny cells coincides with a concomitant increase in binucleates (fusion products) as the population of tiny cells decreases. These, and an extensive amount of other kinetic data, indicate that the tiny amoebae represent the gametes of *Dictyostelium discoideum*.

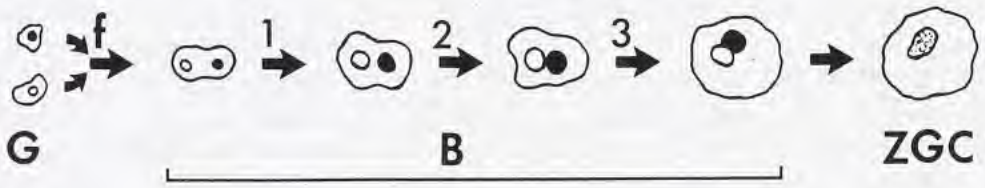

FIG. 1. — Fertilization in *Dictyostelium discoideum*. Shortly after spore germination in dark grown cultures, tiny gametes (G) appear. These actively moving cells make frequent contacts with other cells and upon contacting other gametes they can fuse (f) to form a small binucleate. As the cytoplasm of the binucleate increases in volume, the pronuclei swell (1), migrate together (2) and fuse (3) to form the zygote giant cell (ZGC).

Gametes with essentially identical structure and behaviour, as well as similar developmental kinetics, have been identified in several other genera and species of cellular slime mould including: *D. giganteum*, *D. purpureum*, *D. mucoroides* and *Polysphondylium pallidum* ([26, 27, 28, 52], O'DAY, unpublished results) as well has homothallic strains of *D. discoideum* [RAMA & O'DAY, unpublished]. Thus the gametes of all cellular slime moulds studied to date appear as tiny amoeboid cells possessing tiny nuclei. Preliminary DNA quantification in *D. discoideum* indicates that these cells are haploid and appear to be arrested in G1 of the cell cycle [O'DAY & RIVERA, unpublished results].

Gamete differentiation is strain and calcium-independent

In mated sexual cultures of *D. discoideum* cultivated in the presence of 1.0 mM calcium chloride, the developmental kinetics of gamete differentiation and subsequent fertilization have been well defined [34, 35, 42, 70]. After appearing about 8 hours after culture initiation, the gamete numbers peak at between 10-12 hours after which they steadily decrease in number. The decrease in binucleates coincides with the appearance of their binucleate fusion products which

Source : MNHN, Paris

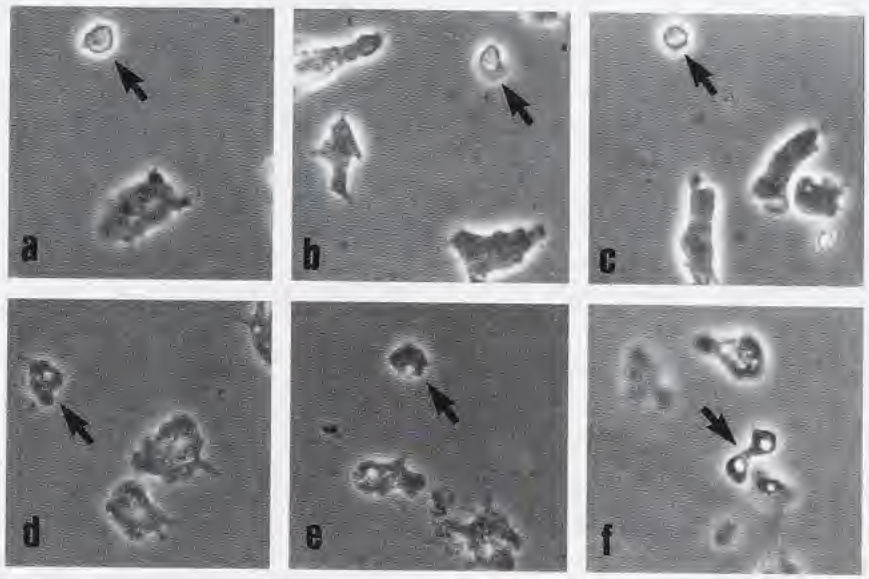

FIG. 2. — Gametes of *D. discoideum in vivo* and after fixation and staining with the nuclear fluorescent dye Hoecsht 33258. a-c, examples of living gametes (arrows) as compared to typical non-sexual amoebae under phase microscopy. After fixation and staining and observation under simultaneous phase and fluorescence microscopy (d-f), the amoebae are seen to contain a small bright nucleus (d, e). Pairs of amoebae (f) also have been caught in the process of fusing.

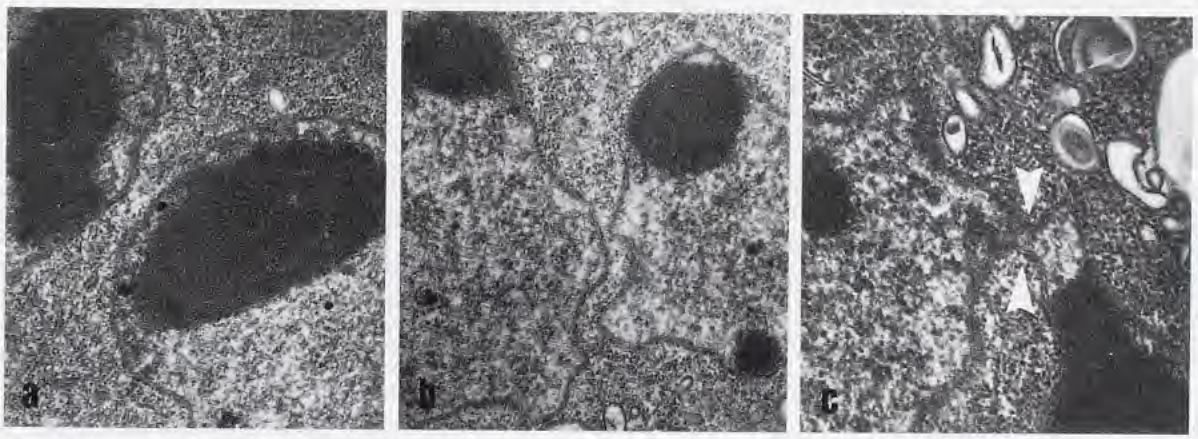

FIG. 3. — Ultrastructure of pronuclear fusion in *D. discoideum*. Pronuclear fusion (a-c) involves and initial contact (b) by protrusions of the swollen pronuclei followed by the fusion of the nuclear envelopes (c) at their points of contact in a manner that is reminiscent of the sea urchin type of fertilization.

then increase in number until about 18 hours. The binucleates are converted to zygotes by events of pronuclear swelling, migration and fusion which are exemplified by the "sea urchin-type of fertilization" [e.g. 33, 51, 69, 70].

In mated cultures grown without the addition of calcium, gametes appear and increase in number to a peak of approximately 30% by 18 hours [42]. However, they do not decrease in number and binucleates do not appear in significant numbers. When strains NC4 or V12 are

Source : MNHN, Paris

grown alone in either the presence or absence of calcium ions, gametes also appear and reach a plateau but do not fuse to form binucleates. If calcium is subsequently added to mixed mating type cultures grown in the absence of added calcium, fertilization is spontaneous and rapid, often resulting in the formation of extremely large, multinucleated cells. The same occurs when strains that have been cultured separately are subsequently mixed together [63, 64]. Gamete formation, then, is independent of an interaction between cells of the opposite mating type. These results reveal that gamete formation occurs in the absence of calcium and that the gametes that form are fertilization-competent. The simple addition of calcium is sufficient to trigger their fusion and all subsequent events of fertilization [42]. What is more, like animal fertilization, the over-abundance of fertilization competent cells leads to polyspermy [42, 55].

Fertilization is calcium dependent and involves signal transduction

While gametes can form in the absence of calcium, they cannot fuse. Calcium is the trigger for fertilization, as well as many other types of biomembrane fusion [53]. During animal fertilization, the events leading to the amalgamation of the sperm and egg are mediated by signal transduction involving calcium ions, calmodulin and its binding proteins (for review see [4, 5, 22]). Various aspects of sperm function in the events leading to fertilization are dependent upon calmodulin function (Table 1). Contact between the sperm and egg, involving a G protein, receptor-mediated process, leads to inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) production within the egg [e.g. 48]. IP3 formed intracellularly mediates the release of calcium ions from membrane bound stores, most notably the calciosomes (e.g. 5, 46). The resulting increase in intracellular calcium concentration leads to an activation of calmodulin followed by the modulation of the activity of several calmodulin binding proteins including CaM-kinase. Simultaneously, DAG is activating protein kinase C (PKC). These downstream events lead to the phosphorylation of certain key proteins as well as other essential, but as yet unclarified, events of fertilization.

Extensive studies during fertilization in *D. discoideum* have shown that the dual calcium signalling pathway mediated by G proteins and involving IP3 and DAG also mediates cell and pronuclear fusion [9, 35]. IP3 augments cell fusion while inhibitors of calcium release from intracellular stores prevent both cell and pronuclear fusion [35]. As in other organisms from *Chlamydomonas* to vertebrates [e.g. 15, 22], inhibitors of calmodulin also prevent gamete cell and pronuclear fusion in *Dictyostelium* (Fig. 4) [36]. In keeping with this, analysis of calmodulin binding proteins (CaMBPs), using a highly sensitive method, revealed over 25 CaMBPs during sexual development of which some developmentally regulated CaMBPs showed developmental kinetics linking them to the events of fertilzation (Fig. 5) [38, 40]. While their identities remain a mystery, two developmental calmodulin binding proteins (CaMBP91 and CaMBP48) are under further analysis. Specific CaM kinase (CaM-Kinase III) and CaM phosphatase activities have also been linked to the events of fertilization in *D. discoideum* (e.g. Fig. 6) [39]. Various studies have shown that calmodulin activity and CaM-dependent protein kinase is involved in nuclear envelope breakdown suggesting a role for this activity in pronuclear fusion [3, 12, 44, 45]. Similarly, pharmacological experments have shown that PKC activity is essential for fertilization and substrates for phosphorylation by this enzyme have been identified [60]. On the other hand, protein tyrosine kinase activity does not appear to be essential [60].

Gamete formation is inhibited by calmodulin

Of particular interest were some unexpected results from these experiments. The addition of the CaM inhibitors, trifluoperazine (TFP) and calmidazolium (R24571) not only inhibited cell and pronuclear fusion but they also led to a dramatic increase in gamete formation (Fig. 4b) [36]. The results were reminiscent of cultures treated with an endogenous regulator of sexual development

Source : MNHN, Paris

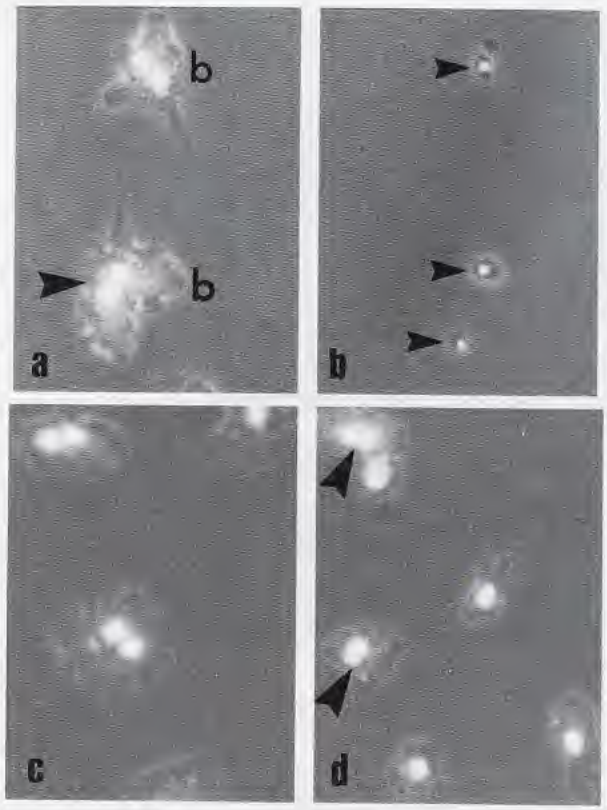

FIG. 4. — Differential effects of calmodulin antagonists on gamete cell fusion and pronclear fusion in sexual cultures of *D. discoideum*. When added at 10 hours, 1.0 μM R24571 yields similar numbers of binucleates (a) as control cultures as seen at 20 hours but 5.0 μM R24571 added at 10 hours completely inhibits gamete fusion (b) and also induces gamete differentiation within the same time period. When added at 18 hours both 1.0 μM TFP (c) and 5.0 μM R24571 (d) inhibit pronuclear fusion as observed in cultures 6 hours later (24 hour old cultures).

called the autoinhibitor [59, 68]. The autoinhibitor is produced and secreted by zygotes apparently as a shut-down mechanism to prevent further fertilization in older sexual cultures. The autohinhibitor, like the inhibitors of calmodulin, inhibited both cell and pronuclear fusion when added to early culture and it similarly augmented gamete differentiation. The autoinhibitor is a small molecular weight (about 500 Daltons), heat stable, hydrophobic molecule [68]. Subsequent studies showed that partially purified autoinhibitor specifically inhibits CaM-dependent phosphodiesterase in a dose-dependent manner [37]. The accumulated information thus indicates that the autoinhibitor functions to inhibit fertilization by inhibiting calmodulin function. It also supports the contention that gamete formation is negatively regulated by calmodulin. Work on *C. elegans* similarly indicates that calmodulin inhibits the onset of spermatogenesis. This may reflect a fundamental role of calmodulin in regulating gametogenesis (Table 1).

Pheromone production by gametes

While several different developmental regulators operate during sexual development of *Dictyostelium discoideum*, at least one other is relevant here. Early work showed that extracellular medium from cultures of NC4 induced V12 to undergo sexual development alone [54]. Continued work showed that a low molecular weight, volatile sexual pheromone was produced by NC4 which induced V12 [27, 41]. On the other hand, strain V12 did not produce detectable levels of sex pheromone under the conditions used. Other species produce sexual pheromones as well and the level of production (i.e., ability to induce other strains of the same species to undergo sexual development) by each strain was directly related to the gamete levels formed by that strain [58].

Source : MNHN, Paris

FIG. 5. — Calmodulin-binding proteins during gamete fusion, pronuclear fusion and zygote differentiation in *D. discoideum*. Cells were isolated at the times indicated and subjected to SDS-PAGE followed by the detection of calmodulin (CaM) binding proteins using the 35S-VU-CaM overlay procedure [29]. More than 25 CaMBPs are present but only seven of these are expressed during sexual development (arrows). A diagrammatic depiction of fertilization is presented for orientation.

Thus four strains of *D. giganteum* which exhibit a hierarchy of strain interactions produce pheromonal activity which is directly related to the number of gametes each strain produces. Three strains of *D. purpureum* which interact in a non-hierarchical manner show the same direct correlation between the production of gametes and the level of pheromone activity. Finally, in *D. discoideum* only NC4 produces pheromone while simultaneously producing the largest number of gametes. Thus the results from several different species reveal a direct relationship between the gametes produced and the level of sexual pheromone activity produced by the strain. This suggests that the gametes are the source of the pheromonal activity. While the pheromone stimulates fusion, no evidence exists as to whether it serves to direct cells of opposite sex together via chemotaxis in an analogous manner to eggs chemoattracting sperm as occurs, for example, in

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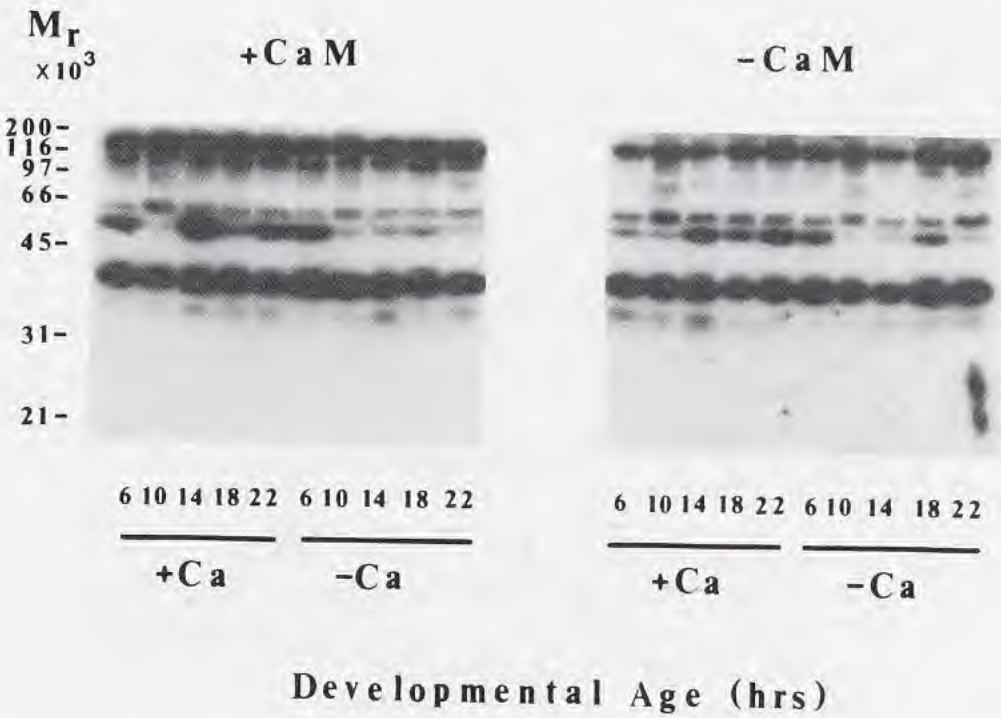

FIG. 6. — Calmodulin (CaM) dependent protein kinase activity during fertilization in *D. discoideum*. While at least 10 different proteins are phosphorylated only two of these (arrows) appear to be preferentially phosphorylated by a CaM dependent protein kinase activity. A high molecular weight protein (upper large arrow) is phosphorylated very little in the absence of CaM while a lower molecular weight protein (lower small arrow) is enhanced to a greater degree in the presence of CaM.

sea urchins via *resact* [76]. So far, the gametes of *Dictyostelium* have proven difficult to purify but their purification could lead to the ability to produce large amounts of pheromone for further investigation which could answer some of the remaining questions about its structure and mode of action. Furthermore, purified gametes would permit the direct analysis of gamete-specific molecules which would allow further analyses of gamete differentiation and its regulation. In spite of this, some information about gamete specific components can be inferred from other experimental approaches.

Glycoprotein, g proteins and the initiation of fertilization

By definition, the function of gametes is to amalgamate in the process of fertilization to generate a new genotype. By extension, the molecules that mediate gamete fusion should be restricted to the gamete cells themselves. Much of the work reported so far is based upon populational studies with cell-type specific macromolecules being identified on the basis of their temporal association with cellular kinetics and cellular or developmental function. In this regard, several glycoproteins have been identified as critical to fertilization and by extension to being components of gametes. Early work showed that certain N-acetylglucosamine-containing glycoconjugates localised at the cell surface were essential for fertilization [8, 56, 62, 67]. Subsequently a glycoprotein of about 130 kDa was identified with the appropriate developmental kinetics, calcium dependence and behaviour after certain treatments (e.g. with tunicamycin [6]).

Source : MNHN, Paris

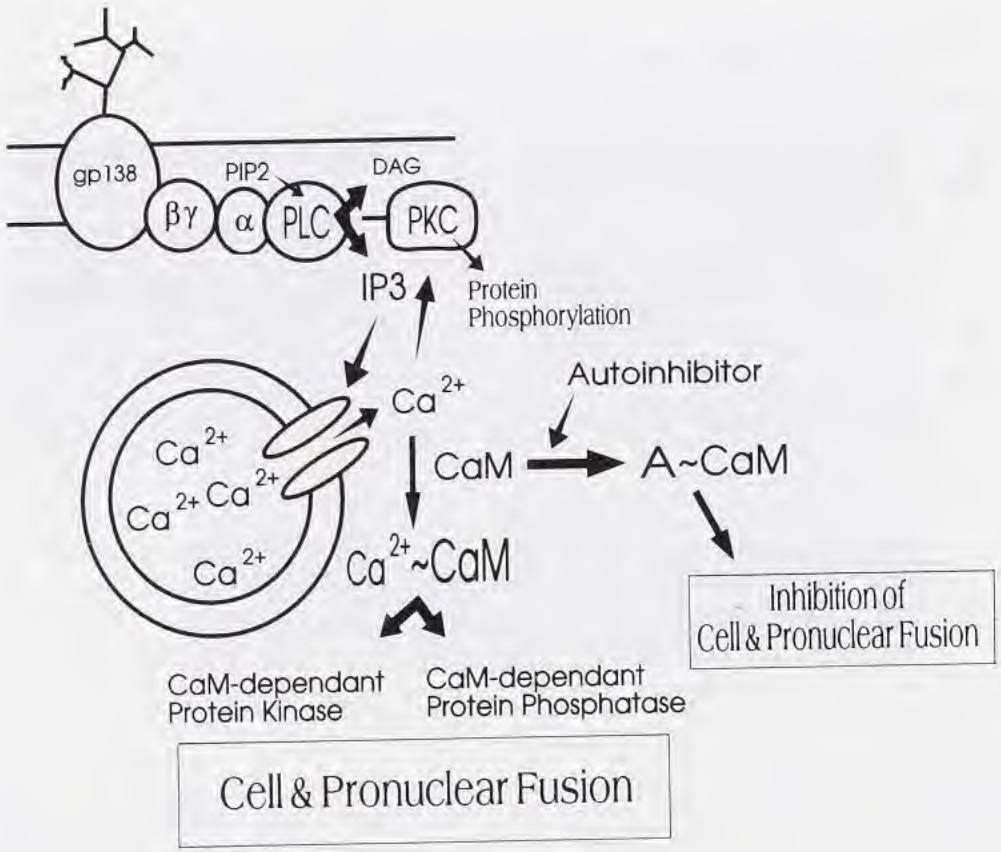

FIG. 7. — Signal transduction during fertilization in *Dictyostelium discoideum*. Cell fusion involves cells of opposite mating type and extensive work has shown that at least one glycoprotein (gp138) mediates the fusion process. Gamete cell fusion involves a G protein mediated process leading to the intracellular increase in the second messenger inositol-1,4,5-trisphosphate (IP3). IP3 leads to an intracellular increase of calcium ions (Ca2+) which then leads to an increase in protein kinase C (PKC) activity and resultant protein phosphorylation. Simultaneously, the Ca2+ binds to calmodulin (CaM) and activates it (Ca2+~CaM). This leads to the activation of at least a CaM-dependent Protein Kinase and Phosphatase. Once cell and pronuclear fusion has produced some zygote giant cells, those cells secrete a low molecular weight autoinhibitor (A) that inhibits CaM and leads to the inhibition of cell and pronuclear fusion.

Almost simultaneously a similar glycoprotein (138 kDa) plus others were identified by Kaichiro YANAGISAWA's group at Tsukuba [65, 67, 75]. YANAGISAWA's group pursued this avenue, leading to the cloning of a gene for gp138 which when used to generate antisense mutants revealed that this glycoprotein was essential for gamete fusion [16]. On the other hand, since it was not strain-specific, their data suggest that while involved in fusion, this gp138 is not a cell type receptor that initiates the fertilization process. Other work indicates that such receptors must exist and awaits further investigation.

While identification of the receptor that initiates the events leading to IP3 and DAG production for fertilization awaits elucidation, other work has identified intermediary components. Membrane-bound heterotrimeric GTPases mediate the interaction between the receptor and its ligand and specific membrane effectors (e.g., phospholipase C leading to IP3 accumulation). Developmental analyses have revealed specific GTPases present during the phases of gamete differentiation and fertilization. Specifically, developmentally regulated, calcium-dependent

Source : MNHN, Paris

GTPases of 52kDa and 45kDA predominate at the time of fertilization [7, 9]. Interference with GTPase function in general by GTP and GDP analogues and of G proteins specifically with aluminum fluoride, argues for the importance of the GTPases in fertilization. Additional inhibitor studies plus probing of western blots with specific antibodies has revealed that a G protein of 52kDa (i.e. dGαs52) is important during sexual phagocytosis but not in fertilization [7].

Conclusions

Our extensive work on early sexual development of *Dictyostelium discoideum* has resulted in the formulation of a working model for the signal transduction events that initiate and terminate cell and pronuclear fusion (Fig. 7). While our studies to date have allowed us to generate an integrated picture of fertilization *in D. discoideum* versus that in animals, many questions remain to be answered. The purification of specific cells types has helped us localize some elements of the signalling process to zygotes but the purification of gametes is essential. Experiments are now underway to generate mutants defective in calmodulin function and to produce knockout mutants for specific CaMBPs [LYDAN & O'DAY, unpublished]. The characterization and sub-cellular localization of specific CaMBPs is currently underway. Several CaMBP genes have been isolated by us from a cDNA library from asexual development. Our goal is to use the sequence information from these CaMBPs to make knockout mutants in homothallic strains of *Dictyostelium* to define which CaMBPs function during fertilization. Continued work should permit us to understand the fundamental events of fertilization in a comparatively simple organism that exhibits the fundamental communication and signal transduction events that occur during fertilization in animals.

ACKNOWLEDGEMENTS

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Source : MNHN, Paris

Sperm and Spermiogenesis of the "Turbellaria" and Implications for the Phylogeny of the Phylum Platyhelminthes

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ABSTRACT

This chapter reviews recent ultrastructural investigations of sperm and spermiogenesis in turbellarian platyhelminths (Catenulida, Nemertodermatida, Acoela, Macrostomida and Trepaxonemata including Polycladida, Lecithoepitheliata, Prolecithophora, Proseriata, Tricladida, and Rhabdocoela, including Typhloplanida, Kalyptorhynchia, Dalyelliida, Temnocephalida and Fecampiida). Some distinctive characteristics of the differentiating spermatid especially in the zone of differentiation may be useful for phylogenetic considerations. Spermiogenetic and mature sperm features including axonemal or flagellar characteristics, dense bodies, nuclear components, mitochondria and microtubule arrangements are documented for each of the major taxa. It is concluded that more investigations are needed and they must be comprehensive to build a solid data base for a cladistic analysis of the phylum based on sperm and spermiogenetic characteristics.

RÉSUMÉ

Spermatozoïdes et spermiogenèse des "Turbellaria" et implications pour la phylogénie de l'Embranchement des Plathelminthes

Les résultats récents sur les spermatozoïdes et la spermiogenèse des Plathelminthes Turbellariés (Catenulida, Nemertodermatida, Acoela, Macrostomida et Trepaxonemata y compris Polycladida, Lecithoepitheliata, Prolecithophora, Proseriata, Tricladida et Rhabdocoela, y compris Typhloplanida, Kalyptorhynchia, Dalyelliida, Temnocephalida et Fecampiida) sont synthétisés. Certaines caractéristiques distinctives de la spermatide en cours d'évolution, spécialement dans la zone de différenciation, peuvent être utiles pour des considérations phylogénétiques. Les caractères suivants de la spermiogenèse et du spermatozoïde sont décrits pour chacun des taxons majeurs: caractéristiques des axonèmes et des flagelles, corps denses, composants du noyau, mitochondries et arrangement des microtubules. La conclusion est que des recherches supplémentaires exhaustives sont nécessaires pour construire une base de données destinée à une analyse cladistique de l'embranchement basée sur les caractères des spermatozoïdes et de la spermiogenèse.

"Turbellaria" is used throughout this review as a convenient term for all the non-neodermatan platyhelminths, i.e. the groups other than Trematoda, Monogenea and Cestoda; it does not imply monophyly. Early studies of spermatogenesis and spermatozoa in Platyhelminthes, based on light microscopy (LM), contained only limited phylogenetic information. Electron microscopy (EM) has proved of greater use. For a recent bibliography of EM of turbellarians, see [74] (also [47, 51]). The last comprehensive review of turbellarian

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Source : MNHN, Paris

spermiogenesis and spermatozoa was published by HENDELBERG in 1983 [25] with further discussion of their phylogenetic significance in 1986 [26]. Turbellarian spermatozoa are considered aberrant since they differ markedly from the supposedly primitive and modified forms found in many other animal groups in association with the primitive mode of sperm transfer, viz. external fertilisation [1, 20, 25, references therein]. They are generally elongate, without distinct head, middle piece and tail, and may be without axonemes, have free flagella (usually two) or axonemes incorporated in the sperm body. In this review we summarise the information currently available from each of the major turbellarian taxa and discuss implications for the phylogeny of the phylum. The classification follows CANNON, 1986 [10], except for the Fecampiida (removed from Rhabdocoela on the basis of ultrastructural and DNA evidence [53]).

OBSERVATIONS

Within each taxon, data on sperm and spermiogenesis will be summarised with reference to the following characteristics:

— Sperm: presence, number, location of axonemes or flagella; presence, arrangement of longitudinal microtubules; nature of nucleus; nature, arrangement of mitochondria; presence, nature of dense bodies.

— Spermiogenesis of flagellate or axonemal sperm: structures in the zone of differentiation (microtubules, rootlets, intercentriolar body, dense plates); changes during elongation (movement of basal bodies, rotation of flagella, fusion of flagella with shaft, etc).

Catenulida

SCHUCHERT & RIEGER [61] described spermiogenesis in *Retronectes atypica* by EM [see also 50]. Ciliary rudiments are present during maturation of sperm (also in *R. sterreri* [15]), associated with conspicuous lamellar bodies. Specialised cell protrusions, interpreted as intercellular bridges, were seen during a restricted early period of spermatogenesis, and at a slightly later stage groups of microvillar protrusions occur along the plasma membrane of the spermatocyte. The spermatozoon has a more condensed nucleus and numerous lamellar bodies, no longer with ciliary rudiments. There are frequent infoldings from the cell membrane and thin branched mitochondria. There appear to be no dense bodies of any kind resembling those in other turbellarian orders.

Nemertodermatida

Only mature sperm of two genera have been examined by EM, *Meara* and *Nemertoderma* [24, 72, 73]. Although modified into an elongate form, the spermatozoa resemble the primitive metazoan type with a distinct head containing the nucleus, middle region where a single axoneme is surrounded by a coiled mitochondrial derivative, and tail where the axoneme extends as a free flagellum. These attributes clearly distinguish Nemertodermatida from all other platyhelminth taxa including the Acoela. The axoneme has the 9+2 microtubule arrangement as in normal body cilia and in the axonemes of many acoel sperm.

Acoela

RAIKOVA & JUSTINE [48] recently summarised data on sperm ultrastructure of 30 species from 11 families, including three investigated in that paper. There are two incorporated axonemes, extending from the proximal end up to, and sometimes into the nuclear region, and a considerable volume of cytoplasm. Sometimes each axoneme lies along the outer edge of a cytoplasmic extension, forming an undulating membrane along each side of the sperm. There are possibly four different arrangements of microtubules in the axonemes of acoel sperm: 9+2, 9+0, 9+1 and 9+"1". The most common configuration is 9+2/9+0, i.e. part of the axoneme has the normal 9+2, with some region(s) lacking the central pair of microtubules (i.e. 9+0) (Fig. 1). Some species

Source : MNHN, Paris

appear to have 9+0 throughout the axonemes. In some species, an axonemal central element consisting of a hollow tube (microtubule?) surrounded by an electron-dense halo has been described, denoted 9+1 [24, 57]. It does not have the double helical arrangement found in the dense central element of Trepaxonemata (see below). A dense central element possibly identical with Trepaxonemata sperm axonemes (9+"1"), was described in *Childia groenlandica* [24] and illustrated in *Mecynostomum auritum* [5]; it is not known whether this element has a double helical structure. RAIKOVA & JUSTINE [48] suggested that a misidentification cannot be excluded for the report of "*Mecynostomum*" with a 9+"1" structure. We have recently found a central dense element with a hollow centre and without double helical structure in *M. auritum* (personal observation).

Individual mitochondria, membrane-bound electron dense bodies of one or two kinds, and cortical and/or central microtubules are present in most species. In *Amphiscolops* [57] spermatids have some peripheral but no internal microtubules, while in the maturing sperm only internal ones were seen (Fig. 1). RAIKOVA & JUSTINE [48] suggest that these two groups of microtubules may be mutually exclusive, and that the internal ones derive from the external ones by an infolding of a longitudinal groove. Several species have been described without any microtubule arrays in the sperm. A fringe of terminal filaments has been observed by LM in two species [22, 27].

Spermiogenesis has been studied by EM in only a few acoel species [e.g. 6, 8, 48, 57]. ROHDE *et al.* [57] observed two centrioles, initially at right angles to each other in the early spermatid. Two free axonemes grow from the spermatid and fuse with the elongating shaft in a distal to proximal direction. The basal body region thus becomes distal. Nucleus, dense bodies and mitochondria migrate into the shaft, the elongated nucleus remaining in the proximal region. There is no intercentriolar body (see below) nor rootlets associated with flagellar basal bodies.

Macrostomida

Most species examined are from Macrostomidae, one from Dolichomacrostomidae (*Paramacrostomum tricladoides*) [22] and two from Microstomidae (*Microstomum* spp. [22]). No appendages are reported in the sperm of the last two families, nor of some species of *Macrostomum* [22] and *Bradynectes sterreri* [49] (both Macrostomidae). A pair of stiff bristles has been described from several species of *Macrostomum* [e.g. 6, 44, 55], and from *Promacrostomum gieysztori* [6]. They are anchored in tapering dense structures in the mid-region of the sperm with a dense, banded region between the base of the rod and the anchor [55]. In cross section, bristles interpreted as modified flagella, consist of a large central dense rod, surrounded by irregular smaller dense rods [55] (Fig. 2). The proximal end of *M. tubum* sperm is split into a fringe of microvilli [44, 55] and there are from 3-6 dense chromatin granules in a row just behind this proximal region (Fig. 3). The remainder of the sperm body is rich in mitochondria and elongate dense bodies with fewer, large dense bodies in close contact with the cell membrane. Two symmetrical, contralateral rows of peripheral microtubules extend along most of the shaft (Fig. 2), each bristle arising from the edge of one of these rows.

Spermatogenesis has been studied by EM only in *M. tubum* [55]. Cells containing numerous sections through chromatin were interpreted as spermatocytes. These gave rise, without further division, to bristles and the lateral rows of microtubules, thus spermatozoa formed directly from spermatocytes. The presence of up to six discrete chromatin granules in the sperm may indicate belated nuclear division or nuclear fragmentation.

Trepaxonemata

The remaining platyhelminth taxa form a monophylum, distinguished by an autapomorphic feature of the sperm axonemes (when present). Instead of the 9+2 arrangement of microtubules found in primitive sperm, the 9 pairs of peripheral microtubules surround a unique central complex (Fig. 6), made up of an electron dense core, a lighter zone around the core and an outer

Source : MNHN, Paris

electron dense zone which, in longitudinal sections, has a double helical structure (Fig. 4). This is known as the 9+"1" arrangement and is unique to this monophylum of the platyhelminths. Many of these taxa exhibit common structures and events during spermiogenesis, resulting in similarities in mature sperm. We will thus describe these basic events, present individual taxa data in tabulated form (Table 1), and note variations or noteworthy aspects separately in the text.

General description of spermiogenesis and spermatozoa in Trepaxonemata. Following spermatogonial and spermatocyte divisions accompanied by incomplete cytokinesis, spermatids usually remain joined in clusters by cytoplasmic bridges or cytophores. The spermatid nucleus moves towards the apical plasma membrane, initially with scattered clumps of chromatin that then become more dispersed and filamentous. The apical region becomes a zone of differentiation (ZD) in which two centrioles develop into the basal bodies of free flagella growing out in opposite directions from one another. A banded structure known as an intercentriolar body (ICB) develops which, when seen between the basal bodies, usually consists of a dense central band with a series of lighter, thinner bands on either side (e.g. Fig. 5). In cross section, the plates are circular, i.e. the structure in three dimensions is a cylinder with discs of various densities. Rootlets, either well developed and sometimes multipartite, or small and indistinct, usually attach to the sides of the basal bodies and extend in the direction of the apex of the nucleus or along the sides of it. Microtubules line the plasma membrane in the ZD. An apical projection, often with electron dense material within it, develops distal to the basal bodies. The main spermatid body elongates, carrying the distal projection and flagella distally (except in *Kronborgia* and Neodermata - see below). The nucleus, with increasingly condensed chromatin, moves into the shaft, along with mitochondria or a single fused mitochondrion, and dense bodies of one or more kinds. Flagella may bend proximally to lie alongside or fuse with the spermatid shaft. In some taxa the flagella also rotate around the shaft so that they emerge adjacent to each other. Aflagellate sperm develop by elongation of the spermatid, lined by microtubules. The nucleus elongates, chromatin condenses and dense bodies and mitochondria become distributed within the shaft.

TABLE 1. — Characteristics of sperm and spermiogenesis of Trepaxonemata (minus Neodermata)

ICB: intercentriolar body; 1 = present; 0 = absent.
R: rootlets or striated structures attached to the proximal sides of basal bodies; 1 = present (D divided); 0 = absent.
DP: dense plates located on the distal sides of basal bodies (may not be homologous in triclads and proseriates; 1 = present; 0 = absent.
ST: axoneme insertion; 1 = axoneme insertion becomes sub-terminal as a result of formation of a distal process, ± = slightly sub-terminal, 0 = no distal process, 1-0 = distal process present but disappears.
BBD: Basal body; 1 = basal body region moves distal during spermiogenesis; 0 = remains proximal.
AN: axoneme number; 2-1 = initially 2 but only one in mature sperm.
AS: axonemes state; F = free, A = adhering; I = incorporated in shaft, I-F = most of axoneme incorporated, short free region.
AP: axonemal placement; A = adjacent to one another; O = on opposite sides of the shaft to one another.
SF: split tips of flagella; 1 = present.
M: mitochondria; M = multiple; R = in rows; D = altered, membranous derivative; F = fused into 1, 2 or 3+ long mitochondria.
NN: nature of nucleus; L = lobed; 2 = 2 components coiled around one another; Rods = dense chromatin rods; E = membranous elaborations; Env = nucleus partially envelops a string of mitochondria.
DB: dense bodies present in main shaft; 1 = present; 0 = absent; 2 = 2 kinds noted.
PM: peripheral microtubules; 1 = present, 1Sp = full peripheral row plus inner spiral group (see text); + = some additional internal microtubules present in some parts; few = widely spaced; inc = incomplete outer row where axoneme(s) are adjacent to the plasma membrane and/or remain outside the ring of microtubules.
G: granules (approximately 25 nm diameter) in conspicuous longitudinal rows beneath the cortical microtubules of the shaft.
Wherever two or more states are separated by a stroke (/), different states were found in different species.
- = structure not present because sperm are aflagellate.

Source : MNHN, Paris

	Characteristics of spermiogenesis					Characteristics of spermatozoon								
Taxon	ICB	R	DP	ST	BBD	AN	AS	AP	SF	M	NN	DB	PM	G
Polycladida														
Cotylea					1	2	F	O		F				
Acotylea	1	1		1	1	2	A	O		MR		1	1	
Lecithoepitheliata														
Prorhynchidae	1	1		1-0	1	2	F/A	O		M		2	1	
Prolecithophora														
(except *Urastoma*)	-	-	-	-	-	0				D	L	0/1	1+	
Urastoma						2	I	A?		F-2		0	1	
Proseriata														
Coelogynoporidae	1	1-D	1	1	1	2	F			M		1	1	
Nematoplanidae	1	1		1		2	F					1	1	
Monocelididae	1	1-D	1	1	1	2	F			MR/F		1	1	
Otoplanidae	1	1	1	1		2	F			MR/F		1	1	
Tricladida														
Terricola	1	1	1	1	1	2	F	A	1	F-1	2	0	1	
Maricola	1	1	1	1	1	2	F	A		F-1	2	0	1+	
Paludicola	1	1	1	1	1	2	F	A	1	F-1	2	0	1+	
Typhloplanida														
Typhloplanidae	1	1/?		±	1	2	I/F	A		M		1	1-inc/1	
Promesostomidae						2	F							
Solenopharyngidae						2	F			MR	E	2?	1	1
Byrsophlebidae	1	1		1	1	2	F	A		MR	Env	2	1	1
Trigonostomidae						0								
Kalyptorhynchia														
Eukalyptorhynchia				±	1	2	I	O		F		2	1	
Schizorhynchia	1	1		±	1	2-1	1			F	Rods	0	1	
Dalyeliida														
Dalyelliidae	1	1-D		1	1	2	F	A		MR	Env	2	1	
Provorticidae						0								
Graffillidae	?/-/1	?/-/1		1/-/±	1/-/1	2/0/2	F/-/1	?/-/A		?/F-2/R		1/0/2	1/1/1-inc	1
Luridae	-	-	-	-	-	0				M		1	1 few	
Umagillidae	1/	?/			1/-	2/0	F/-	O	1/-	M		1	1	
Pterastericolidae	1 (weak)	0		0	1	2	I-F	O		M		1	1-inc	
Temnocephalida														
Temnocephalidae	1	1		1	1	2	F	A		M		1	1Sp	1
Actinodactylellidae	1	1		1	1	2	F	A		M		1	1Sp	
Scutariellidae	1	1		1	1	2	F	A		F-2		0	1	1
Didymorchidae	1	1		1	1	2	F	A	1	M		1	1Sp	1
Fecampiida														
Fecampiidae	0	0	0	0	0	2	I	O		F-3+		0	1	

Source : MNHN, Paris

Polycladida

There are many LM studies of Polycladida [e.g. 21, 22], but only one EM study of spermiogenesis (*Notoplana japonica*) [38] and few EMgraphs of sperm [e.g. 27]. Flagella remain free in Cotylea but superficially united with the shaft in Acotylea, sometimes part of an undulating membrane but never wholly incorporated beneath the plasma membrane. Flagella arise from and remain on opposite sides of the shaft very close to the distal tip of the spermatozoon [22]. There are multiple dense bodies [e.g. 27] and multiple mitochondria, some apparently fused into larger aggregates [38]. Longitudinal microtubules lie in a complete ring beneath the plasma membrane, apparently for the whole length of the spermatozoon.

Spermiogenesis in *Notoplana japonica* [38] is basically as described above for the Trepaxonemata. KUBO-IRIE & ISHIKAWA [38] described additional proximal centrioles attached at right angles to the main ones during spermiogenesis, but the micrographs are not clear. There has been no other report of four centrioles in the ZD of a platyhelminth spermatid.

Lecithoepitheliata

There is one EM study of sperm and spermiogenesis of *Prorhynchus* sp. [79] (Table 1). Sperm have an irregular contour, and flagella lie in grooves of wide lateral extensions of the shaft (Fig. 13). The testes of some Baikal endemic *Geocentrophora* species (*G. intersticialis*, *G. wagini* and *G. wasiliewi*) were examined [7] and sperm found to have two free flagella with 9+"1" axonemes, longitudinal microtubules and 1-5 longitudinal folds. No members of Gnosonesimidae have been investigated.

Prolecithophora

Mature sperm have been examined in several species [e. g. 11, 14, 16, 22, 43, 60] and spermatid development in *Multipeniata* [60]. Sperm are aflagellate, have a row of cortical microtubules, lack dense bodies (except for *Multipeniata)* and have a complex intraspermial membranous system (mitochondrial derivative?) closely associated with the lobed nucleus in a spiralling arrangement in some species. Sperm of *Urastoma cyprinae* do not resemble those of the other prolecithophorans examined [46]. There are two initially free axonemes that become completely incorporated in the mature sperm body, peripheral microtubules that are crowded into the centre of the shaft at one end, at least two mitochondria of regular appearance, no dense bodies and no membranous system. It is possible that *Urastoma* may not belong to the Prolecithophora, as suggested by NOURY-SRAÏRI *et al* [46].

Proseriata

There are several reports and a few micrographs of some aspects of sperm [22, 25, 65, 69] and three reports of some aspects of spermiogenesis from three of the seven families [65-67, 69]. Sperm have two free, sub-terminally inserted axonemes, peripheral microtubules, an elongate nucleus, numerous dense bodies, and mitochondria arranged in a tightly packed longitudinal row or rows (Fig. 4). During spermiogenesis, a well developed ICB lies between the basal bodies, and rootlets (sometimes multipartite) extend from the basal bodies towards and alongside the nucleus. In three species of Parotoplaninae (Otoplanidae), a distinctive striated distal appendage to the ICB forms during cell elongation. SOPOTT-EHLERS [67] suggested the structure to be an autapomorphy of Parotoplaninae. Changes in the ICB were also documented in a nematoplanid [66] and a coelogynoporid [65, 69], involving considerable stretching and partial splitting in the first, and transformation of some elements and partial splitting in the second.

Source : MNHN, Paris

Tricladida

Species from all three sub-taxa (Paludicola, Maricola and Terricola) have been examined by EM [17-19, 28, 30, 31, 37, 40, 59, 62, 63] and some common characteristics identified [59]. All have two free, sub-terminally inserted flagella which arise together on one side of the shaft, a distal projection beyond the flagellar insertion, and nucleus and a single fused mitochondrion (Fig. 8) extending throughout most of the remaining length of the sperm (in contrast to many other turbellarians, in which the nucleus is restricted to a shorter proportion of the sperm body). The nucleus has two distinct components, one (the protein component [62]) less dense than the other, coiled around each other in screw-like fashion (Figs 6, 8). In at least some species, nucleus and elongate mitochondrion also coil around each other. Flagella of mature sperm contain many small granules and the tips split into smaller units (Fig. 6) (Paludicola, Terricola). Mature sperm have a short inner row of microtubules in addition to the full peripheral row (Paludicola, Maricola) and lack the numerous dense bodies characteristic of many turbellarians (Paludicola, Maricola, Terricola). During spermiogenesis, the nucleus has a dense apical layer, there are short cross-striated rootlets from the basal bodies to the nucleus, and dense, half-moon shaped plates around the basal bodies opposite the rootlets (Fig. 5) (Paludicola, Maricola, Terricola). In at least two paludicolans and one maricolan the ICB splits into two halves attached to the basal bodies during rotation [18, 59].

Rhabdocoela

Typhloplanida. There are LM reports of sperm with and without free flagella [e. g. 22], but only detailed EM reports of three species, *Bothromesostoma personatum* [13] and *Phaenocora anomalocoela* [80], both Typhloplanidae, and *Anthopharynx sacculipenis*, Solenopharyngidae [68]. *B. personatum* sperm have two free, subterminally inserted flagella emerging together on the same side of the shaft, dense bodies, mitochondria and a complete ring of cortical microtubules. During spermiogenesis, dense heels form at the ends of the basal bodies which then rotate around the shaft, resulting in compression of one semicircle of microtubules into a tight double row. *P. anomalocoela* sperm (Fig. 12) have two fully incorporated axonemes of unequal length, numerous mitochondria and dense bodies. Basal bodies are close together and axonemes also remain adjacent for their entire length, situated between the plasma membrane and the row of microtubules. The nucleus extends almost the entire length of the sperm. During spermiogenesis, dense heels form at the ends of basal bodies, and massive rootlets are attached to the sides of basal bodies as they rotate around the shaft, compressing some of the peripheral microtubules into a central double row. The manner in which the compressed row rejoins the outer microtubules does not result in a spiral formation (cf. Temnocephalida below). *A. sacculipenis* sperm are filiform, totally enclosed by cortical microtubules, possess two free flagella, one or two rows of tightly packed mitochondria, and have regular rows of granules beneath the plasma membrane. There are numerous dense bodies along the shaft and terraced elaborations of the nuclear membrane. Similarly, two free flagella, regular rows of granules, dense bodies and a tightly packed row of mitochondria are present in the sperm of *Maehrenthalia* sp. (Byrsophlebidae) [75]. *Typhloplana virida* has two free, 9+"1" flagella [42]

Kalyptorhynchia. By LM and EM, no sperm have been found with free flagella [22, 25, 39].

— *Eukalyptorhynchia*. EMgraphs of sperm of *Gyratrix* sp. (Polycystidae) and *Odontorhynchus* sp. (Fig. 14) (Gnathorhynchidae) [52] and *Polycystis naegelii* (Polycystidae) [39] show dense bodies, probably of two kinds, an elongate mitochondrion, peripheral microtubules and two fully incorporated axonemes. In *P. naegelii* the nucleus contains a number of dense bodies (long rods or short rounded structures?). Basal bodies are staggered and slightly

Source : MNHN, Paris

subterminal. L'HARDY [39] also studied spermiogenesis in *Polycystis naegelii* - initially free flagella are incorporated in a distal to proximal manner.

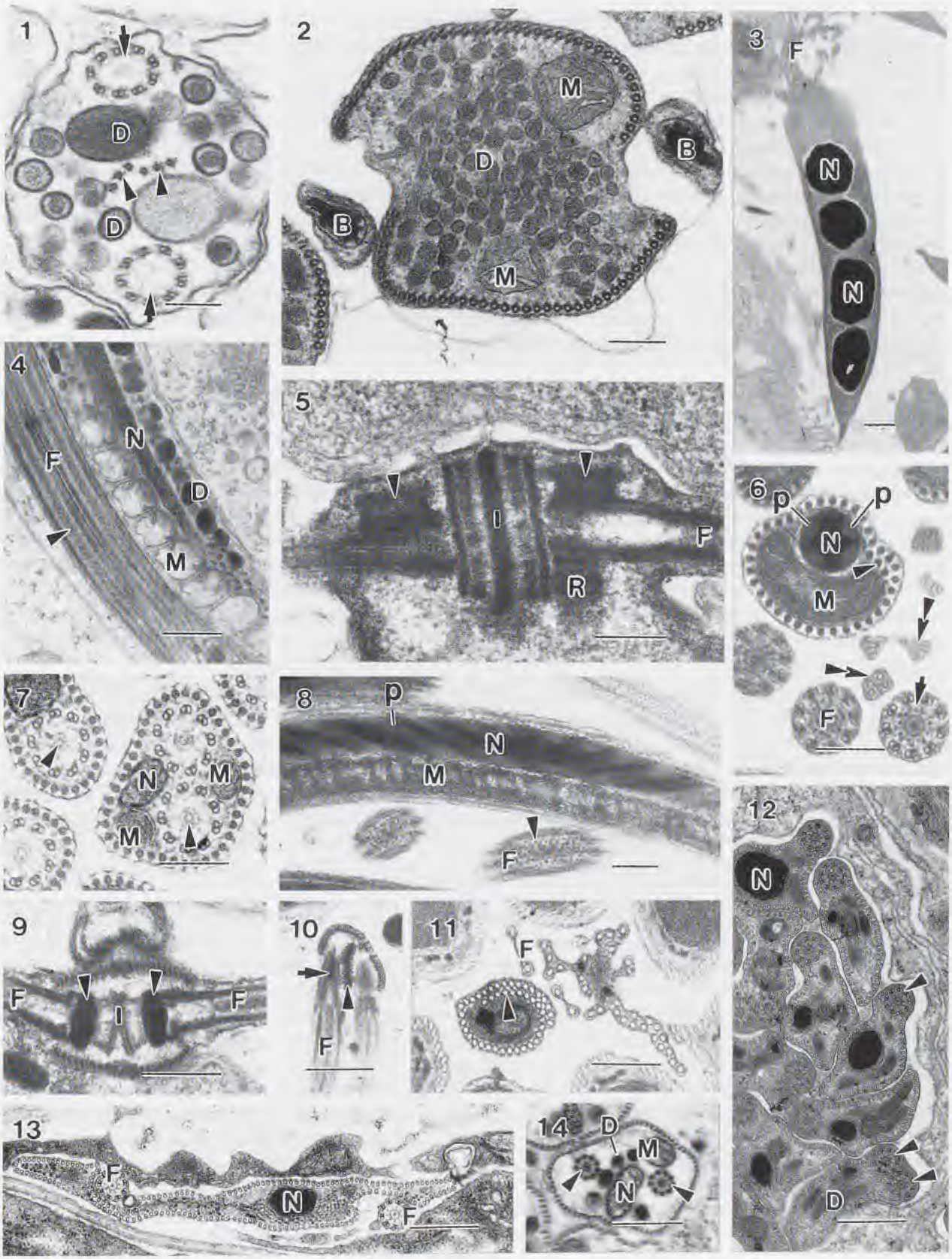
— *Schizorhynchia. Baltoplana magna* sperm have a single incorporated axoneme [23, 25]. WATSON & L'HARDY [76] found two basal bodies in the ZD, with an ICB between them. Only one develops into a normal flagellum - the other remains as a short bud. Both are carried distally by the elongating shaft and one is incorporated into the shaft in a distal to proximal direction while the spermatid is still pyriform. The other degenerates and mature sperm have only a single axoneme, from one end to almost the other end. The shaft is surrounded by cortical microtubules, there is an elongate mitochondrion, no dense bodies and the nucleus has an unusual arrangement of long dense chromatin rods. Preliminary observations by SCHOCKAERT (personal communication) suggest that several schizorhynch families may contain taxa with monoaxonemal sperm.

Dalyelliida. This diverse group contains free living, symbiotic, and parasitic species, but there are few reports of detailed EM investigations.

— *Dalyelliidae.* Two micrographs of sections of *Microdalyellia* sperm [27] and a full study of spermiogenesis and sperm of *Gieysztoria* sp. [82] show two free axonemes, multiple mitochondria, dense bodies (2 kinds in *Gieysztoria*), and in *Gieysztoria* the nucleus almost completely envelops a string of mitochondria along the shaft. During spermiogenesis in *Gieysztoria*, an ICB lies between the basal bodies, dense heels develop at the bases of basal bodies, and flagella rotate around the shaft to lie adjacent to one another. Rotation causes compression of one semicircular row of peripheral microtubules, so that they lie in a double row in the middle of the spermatid, in the region of the basal bodies. The manner in which the compressed row rejoins the outer row does not result in a spiral formation (cf. Temnocephalida).

FIGS 1-14. — Spermatozoa of "Turbellarian" Platyhelminthes. **1:** Transverse section of sperm of *Amphiscolops* sp. (Acoela). Note incorporated axonemes (arrows) without central element, two sizes of dense bodies (D), central array of microtubules (arrowheads). Bar = 200 nm. **2:** Transverse section of sperm of *Macrostomum tubum* (Macrostomida). Note numerous dense bodies (D), mitochondria (M), bristles (B) and contralateral rows of peripheral microtubules. Bar = 200 nm. **3:** Longitudinal section of sperm of *Macrostomum tubum* (Macrostomida). Note terminal fringe of filaments (F) and separate nuclei or nuclear fragments (N). Bar = 500 nm. **4:** Longitudinal section of sperm of *Monocelis* sp.(Proseriata). Note nucleus (N), dense bodies (D), row of mitochondria (M) and flagella (F) with double-helical central element. Bar = 200 nm. **5:** Zone of differentiation of *Romankenkius libidinosus* (Tricladida). Note intercentriolar body (I), rootlet (R), flagellum (F) and dense plates (arrowheads). Bar = 200 nm. **6:** Transverse section of sperm of *R. libidinosus* (Tricladida). Note nucleus (N) with dark and lighter (p) components, some additional internal microtubules (arrowhead), free flagellum (F), split tips of flagellum (double arrowheads) and dense granules between spokes of flagellum (arrow). Bar = 200 nm. **7:** Transverse section through sperm of *Kronborgia isopodicola* (Fecampiida). Note nucleus (N), mitochondria (M), and incorporated axonemes with hollow central elements (arrowheads). Bar = 200 nm. **8:** Longitudinal section of sperm of *R. libidinosus* (Tricladida). Note single long mitochondrion (M), nucleus with spiralling lighter component (p), and flagellum (F) with dense granules (arrowhead). Bar = 200 nm. **9:** Zone of differentiation of *Decadidymus gulosus* (Temnocephalida). Note intercentriolar body (I) beginning to split, and dense heels (arrowheads) at the ends of the basal bodies of flagella (F). Bar = 500 nm. **10:** Spermatid of *Decadidymus gulosus* (Temnocephalida) after rotation of flagella. Note inner compressed double row of microtubules (arrowhead) and spur on the basal body (arrow) of a flagellum (F). Bar = 500 nm. **11:** Cross sections through sperm of *Craspedella spenceri* (Temnocephalida). Note spiral microtubule arrangement (arrowhead) and filaments (F) resulting from splitting of the proximal end of the sperm. Bar = 200 nm. **12:** Cross sections through sperm of *Phaenocora anomalocoela* (Typhloplanida). Note nucleus (N), dense bodies (D) and one or two axonemes (arrowheads) incorporated just beneath the plasma membrane. Bar = 500 nm. **13:** Transverse section of sperm of *Prorhynchus* sp. (Lecithoepitheliata). Note nucleus (N) and flagella (F) lying in grooves of the shaft. Bar = 500 nm. **14:** Transverse section through sperm of *Odontorhynchus* sp. (Kalyptorhynchia). Note nucleus (N), dense body (D), single mitochondrion (M) and incorporated axonemes (arrowheads). Bar = 500 nm.

Source : MNHN, Paris



Source : MNHN, Paris

— *Provorticidae. Provortex balticus* has elongate sperm, without flagella [22].

— *Graffillidae*. The sperm of the parasitic species *Paravortex cardii*, *P. karlingi*, *P. tapetis* and *Graffilla buccinicola* are aflagellate [12, 22, 45] while free-living *Pseudograffilla arenicola* has two free, sub-terminally inserted flagella [22]. In the three *Paravortex* species, regular longitudinal rows of small dense granules extend throughout the sperm, beneath the peripheral microtubules. They resemble regular rows found in temnocephalans and do not react positively to the Thiéry test for glycogen [45]. Similar rows of granules (also negative to the Thiéry test) are present in the sperm of *Bresslauilla relicta* [75] which have two superficially attached 9+"1" axonemes, two kinds of dense bodies (one occurring outside of the cortical microtubules) and a single row of mitochondria. NOURY SRAÏRI *et al.* also found small numbers of membrane-bound dense bodies in *P. cardii* and *P. tapetis* [45].

— *Luridae* [70]. In *Luriculus australiensis* [58] microtubules in a helical configuration surround the spermatid, and progressive elongation results in formation of an aflagellate, filiform spermatozoon, containing regular rows of dense bodies, mitochondria and a ring of a small number of longitudinal peripheral microtubules, interrupted where organelles are close to the surface.

— *Umagillidae*. From LM, *Syndesmis echinorum* sperm are reported to be aflagellate [22]. *Cleistogamia longicirrus* and *Seritia stichopi* sperm (EM) [56] have two free flagella, numerous dense bodies, multiple mitochondria and peripheral microtubules. *Syndisyrinx punicea* has similar sperm (and split flagellar tips) [54], an ICB is present during spermiogenesis, and although dense floccular material surrounds the basal bodies, no rootlets were seen [41].

— *Pterastericolidae*. Sperm of 5 species of *Pterastericola* and spermiogenesis of one of these (*P. astropectinis*) have been examined by EM [33, 83]. A faint ICB is present but apparently no rootlets. Basal bodies and free flagella are carried distally at the end of the elongating spermatid, before fusing with the shaft for most or all of their length. There are a few dense bodies, a small number of proximally positioned, elongate mitochondria and a row of peripheral microtubules which is discontinuous where one or sometimes both axonemes lie close to the surface. Regular centrioles composed of triplets were seen in mature sperm.

Temnocephalida. Sperm and spermiogenesis have been studied in one species from each of Actinodactylellidae, Scutariellidae and Didymorchidae and at least 12 from Temnocephalidae.

— *Temnocephalidae*. There are more or less comprehensive reports of sperm and spermiogenesis in seven species of *Temnocephala*, three of *Craspedella,* and *Decadidymus gulosus* and *Diceratocephala boschmai* [36, 82, 84-87]: they are compared by WATSON & ROHDE [82]. They have in common the formation of dense heels on the basal bodies (Fig. 9) and compression of a semicircle of microtubules by rotation of the basal bodies around the shaft as described above for *Gieysztoria*. However, the two edges of the compressed row rejoin the uncompressed row in an unequal manner, resulting in a region of the spermatid and mature sperm in which the peripheral microtubules are arranged spirally (Fig. 11). In addition, proximal ends of mature sperm have either a terminal flange of microtubules within the plasma membrane, or are split into a fringe of fine filaments containing microtubules (Fig. 11). It is likely that flange and split shaft represent modifications of the one feature, since both structures occur in the one genus (*Temnocephala*) [84] and there is a tendency for the flange in *T. dendyi* to break up into filaments (personal observation). Membrane-bound dense bodies and numerous smaller granules, in regular rows beneath the microtubules, are present in the sperm of most of the species examined. A dense body is usually located very close to the end microtubule of the inner spiral row.

— *Actinodactylellidae*. Spermiogenesis in *Actinodactylella blanchardi* [82] involves formation of an ICB, rootlets and a similar dense heel on the basal bodies, flagellar rotation and compression of one semicircular row of microtubules and a spiral region of microtubules where the compressed region rejoins the other microtubules. The proximal end of the mature sperm is split into a fringe of filaments, containing microtubule(s).

Source : MNHN, Paris

— *Scutariellidae*. Sperm of *Troglocaridicola* sp. have a similar proximal fringe of filaments and dense heel formation at the ends of basal bodies during spermiogenesis [29] and compression of a semicircular row of microtubules by rotation of the basal bodies. However, expansion of this row apparently does not result in a spiral arrangement. Mature sperm have regularly arranged longitudinal rows of small granules beneath the peripheral microtubules, similar to those found in *Paravortex* (Graffillidae) and Temnocephalidae, but no membrane-bound dense bodies. The distal, anterior region comprises an electron dense, not membrane-bound corkscrew originating from the apical process of the spermatid. This structure appears to be unique among the free-living platyhelminths but resembles terminal structures found in many cestodes [3] and the monogenean *Calicotyle* [71, 81].

— *Didymorchidae* [32]. Only *Didymorchis* sp. (from *Cherax destructor*) has been examined [82]. Sperm and spermiogenesis are as in *Actinodactylella*; the nucleus in the distal region also has a thin trailer which extends to the plasma membrane (also present in *Temnocephala minor* [84]).

Fecampiida. Kronborgia isopodicola is the only species in which sperm or spermiogenesis has been examined [77, 78, 88]. Sperm have two fully incorporated axonemes of unequal length, longitudinal, peripheral microtubules, no dense bodies and a small number of elongate mitochondria (Fig. 7). Spermiogenesis [78] involves formation of two free axonemes, incorporated into the spermatid in a proximal to distal manner. There is no ICB nor are there flagellar rootlets, but many microtubules attached to the basal bodies probably effect rotation of the basal bodies to lie parallel from their initial orientation at right angles to each other. Both ends of the sperm taper markedly, the proximal (anterior) end containing a dense rod which becomes horseshoe shaped in cross section and in which the ends of the longitudinal microtubules are embedded. The distal (posterior) end beyond the nucleus consists of a long narrow filament containing a few, and eventually no microtubules.

Phylogenetic implications

The validity of comparisons of sperm and spermiogenesis of different taxa depends on quality of fixation and "completeness" of the investigations. Concerning fixation, clarity of most cellular components differs in different studies even if specimens are processed similarly. For example, dense bodies sometimes appear very dense and/or membrane-bound and sometimes very indistinct. Reasons could be real differences in composition, fixation artefacts or changes with development. Smaller, not membrane-bound granules are sometimes noted in the cytoplasm of the sperm body (Table 1) arranged in very regular rows, best seen in tangential longitudinal section. Their absence in other groups could be of phylogenetic significance or a fixation artefact.

Concerning "completeness" of investigation, many studies were carried out to answer particular questions, such as whether sperm are biaxonemal and have the 9+"1" arrangement. Such brief investigations allowed some important generalisations leading to the establishment of the taxon Trepaxonemata, the distinction between Cotylea and Acotylea sperm, and recognition of the complete incorporation of axonemes in kalyptorhynchs (indicating monophyly). Some studies are less complete than others because few cells of a particular stage were available or because serial sectioning was not used. Thus it is unclear whether certain structures such as rootlets, ICB, spiral of microtubules etc. were overlooked or are indeed absent. When comparing the morphology of the nucleus, for example, degree of maturation of the spermatid is crucial and sperm should be examined from the seminal vesicle or sperm ducts as well as from the testis.

Considering all this, we must conclude that only a small number of species has been examined in appropriate depth for detailed phylogenetic considerations. Suitable data can be used in two ways for such considerations - to examine the monophyly of established taxa, and to look for evidence of relationships between such taxa. As a first step, autapomorphic feature states need to be identified (refer to Table 1).

Source : MNHN, Paris

1. Flagella: presence, kind, number and location. The primitive metazoan sperm is considered to be monoflagellate [20], while most platyhelminths possess two flagella or incorporated axonemes. The biaxonemal condition could be plesiomorphic for the whole phylum (lost in Catenulida and Nemertodermatida, highly modified in Macrostomida) or independently acquired in Acoela and Trepaxonemata (or in Acoela and Rhabditophora if the bristles of macrostomids are modified flagella). Absence of flagella, reduction to a single flagellum, and the state of incorporated axoneme(s) are undoubtedly apomorphic states in some taxa, and the unique central element is a synapomorphy for Trepaxonemata (unless the central element in some acoels also has the same structure, but see earlier note regarding possible misidentification). All Acoela, Kalyptorhynchia and Neodermata have axonemes incorporated in the sperm body, all acotylean polyclads have axonemes superficially attached to the sperm body, and all Prolecithophora (except *Urastoma*) lack axonemes. Paired incorporated axonemes in Acoela are not associated with the presence of an ICB as they are in Kalyptorhynchia and Neodermata, and are therefore likely to be autapomorphic. Incorporated axonemes are also found sporadically in other taxa (eg. Pterastericolidae, *Phaenocora* in Typhloplanidae, *Bresslauilla relicta* in Graffillidae), where they represent a derived condition relative to other species in the taxon. The same applies to aflagellate sperm. Thus, all Catenulida and Prolecithophora (except *Urastoma*) lack flagella, but so also do some species of rhadocoels, e.g. the only species of Luridae and Provorticidae examined, four of the six species of Graffillidae examined, and one species of Trigonostomidae. With so few taxa examined in detail, we cannot imply relationships between higher taxa on the basis of presence, absence, incorporation or not of flagella, especially since loss of structures is common in evolution and these traits may be more closely linked to reproductive biology than to ancestry. Table 1 shows that flagella arise on opposite sides of the shaft in most taxa but adjacent to each other in Tricladida, Dalyelliidae, Temnocephalida and Typhloplanidae. In the last three the condition is likely to be synapomorphic, since it is associated with a unique mode of rotation of the flagella during spermiogenesis; it may be of independent origin in Tricladida because different structures are present in the ZD.

In at least four species, all symbiotic, *Syndisyrinx punicea*, *Kronborgia isopodicola*, *Pterastericola asamushii* and the monogenean *Anoplodiscus cirrusspiralis*, the central element of axonemes appears hollow in cross section instead of solid as in other trepaxonematan taxa, although it does have the double helical arrangement in longitudinal section. The phylogenetic significance of this difference is unknown.

2. Mitochondrion/mitochondria. Within the phylum a wide spectrum of mitochondrial formations is present ranging from numerous small mitochondria, through regular, tightly packed rows of individual mitochondria, to varying degrees of fusion and formation of mitochondrial rods and derivatives. The fused forms derive from many small mitochondria during spermiogenesis. Acoela all retain scattered individual mitochondria, triclads and kalyptorhynchs appear to have a single rod-like mitochondrion, prolecithophorans (except *Urastoma*) have a complex membranous derivative, at least two families of Proseriata have regular rows of tightly packed mitochondria, whereas within the Dalyelliida and Temnocephalida a range of states occurs. Presence of small and of fused mitochondria can be demonstrated, but it is very difficult to prove that only a single mitochondrial rod exists because of the considerable length of many sperm. It is likely that fused mitochondria have arisen many times independently. However, if it can be shown that *all* species in the taxon exhibit the derived state (such as in Prolecithophora (except *Urastoma* which may not be a prolecithophoran), Neodermata, Tricladida) then it is likely to be an autapomorphy for that taxon, especially if there are other sperm or spermiogenetic synapomorphies for the group.

Among other characters of sperm and spermiogenesis, a number of apomorphic states can be identified and used for phylogenetic deliberations. These are as follows:

a. The 9+"1" axoneme in Trepaxonemata (Polycladida, Lecithoepitheliata, Proseriata, Tricladida, Typhloplanida, Kalyptorhynchia, Dalyelliida, Temnocephalida and Neodermata).

Source : MNHN, Paris

Prolecithophora are aflagellate (except *Urastoma*), therefore indeterminate on this basis. Macrostomids are aflagellate or have a pair of stiff bristles, perhaps modified flagella, but there is no 9+"1" structure to suggest inclusion in this taxon, although secondary modification cannot be excluded. Some acoel species have a solid central element but its homology with 9+"1" has not been established;

b. The ICB is present in most species of Trepaxonemata possessing axonemes (notably absent in *Kronborgia* and monopisthocotylean monogeneans [35]). It does, however, appear to vary in composition and in the changes which it undergoes during spermiogenesis in different taxa, and these variations may be autapomorphic for smaller taxa;

c. Helical arrangement of two distinctive components of the nuclei of triclads;

d. Lobed nucleus of prolecithophorans;

e. Spiral arrangement of microtubules in the region of sperm adjacent to the flagellar insertion in a taxon consisting of Temnocephalida minus Scutariellidae;

f. A proximal terminal flange or fringe of microvilli-like processes on mature sperm of Temnocephalida. A terminal fringe is also present in some *Macrostomum* sp. and two acoels, but clear differences in other sperm characters suggest independent origin in these groups;

g. A suite of characteristics seen during spermiogenesis in Temnocephalida, Dalyelliidae, Byrsophlebidae and Typhloplanidae (dense heel formation at the end of basal bodies, rotation of flagella around the shaft resulting in compression of a semicircle of microtubules, formation of a spur on the basal bodies in mature sperm). In *Decadidymus gulosus* (Temnocephalidae) the ICB splits completely and one half accompanies each basal body during rotation - it is likely that the same occurs in the other taxa. This combination of characteristics suggests a common ancestor for these groups. It is not present in Pterastericolidae and Umagillidae where it may have been secondarily lost or the absence could indicate that these families are misplaced. Some dalyelliid families have species with aflagellate sperm, hence no ICB etc., some have not been studied, and no other typhloplanid families have been examined. It is therefore premature to draw phylogenetic conclusions, but it does appear that detailed studies of events in the ZD of many more species could help to clarify relationships within Rhabdocoela (minus Neodermata). The ICB also splits into two in some triclad species, but different accessory structures are involved;

h. The bristles, multiple nuclei and unusual spermiogenesis of *Macrostomum* have not been seen in any other platyhelminth taxa and also differ significantly from the primitive sperm and spermiogenesis models of many Metazoa: they must be seen as apomorphies.

The foregoing discussion indicates that sperm and spermiogenetic characters support the monophyly of each of the Acoela, Polycladida Acotylea, Prolecithophora (except *Urastoma*), Tricladida, Temnocephalida minus Scutariellidae, Kalyptorhynchia, Neodermata. They also provide significant evidence to suggest that some taxa may be misplaced, e.g. *Urastoma* may not be a prolecithophoran and *Kronborgia* is not a rhabdocoel.

Regarding relationships between higher taxa, an area of great interest concerns the origin of the Neodermata, without doubt a monophylum as shown by a range of characteristics including the following: sperm with completely incorporated axonemes (or single axoneme or none by secondary reduction), a single elongate mitochondrion (or none by reduction), no dense bodies, and incorporation of axonemes in the proximal to distal direction [4, 35]. Suggested sister group relationships to Neodermata have been proposed on the basis of the common occurrence of one or more of these character states in some turbellarian groups (in conjunction with other characters not related to sperm and spermiogenesis), e.g. with Fecampiidae [77] and with a clade comprising Pterastericolidae, Fecampiidae and Acholadidae [34]. However, the first has not been supported by DNA studies and at least the inclusion of Pterastericolidae in the second is not supported by the later finding of dense bodies and distal to proximal fusion in spermiogenesis of Pterastericolidae [83]. There is also no evidence from sperm or spermiogenesis in support of a close relationship of Temnocephalida and Neodermata as suggested by BROOKS [9], or of "Dalyellioida" and

Source : MNHN, Paris

Neodermata suggested by EHLERS [15]. The sister group of the Neodermata remains undetermined.

The question of the relationship between Catenulida, Acoelomorpha and Rhabditophora has been addressed by several authors [e.g. 2, 15, 64 and references in these] but recent studies of sperm and spermiogenesis only confirm the divisions, without shedding light on their interrelationships. Spermatogenesis and spermatozoa in the Catenulida are unlike those observed in any other platyhelminth group. If the monoflagellate condition of Nemertodermatida is plesiomorphic (i.e. if the last common ancestor of the Acoelomorpha was monoflagellate with a 9+2 flagellum), then presence of two flagella in Acoela and the remainder of the platyhelminths is likely to be of independent origin. However, if a second axoneme has been lost in Nemertodermatida, the biflagellate condition need only have arisen once in the stem species of Acoelomorpha and Rhabditophora. Some sperm with two axonemes have been found in *Nemertoderma* sp. A [73] but spermiogenesis has not been studied.

The taxa commonly known as rhabdocoels (Typhloplanida, Kalyptorhynchia, Dalyelliida and Temnocephalida) appear from DNA and protonephridial ultrastructure to constitute a monophylum, but relationships between the orders and placement of some families within orders are uncertain. JONDELIUS & THOLLESSON [34] used parsimony analysis of 21 LM and EM characteristics to derive a working hypothesis for the Rhabdocoela, but the homology of many of the characters utilised is questionable. Sperm and spermiogenesis studies may prove useful because certain taxa within the group have very distinctive structures and processes.

In conclusion, sperm and spermiogenesis data have the potential to contribute significantly to phylogenetic analysis within the turbellarians, but many more subordinate taxa must be examined for a parsimony analysis.

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Source : MNHN, Paris

Spermatozoal Ultrastructure and Phylogeny in the Parasitic Platyhelminthes

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ABSTRACT

The ultrastructure of spermiogenesis and spermatozoon is briefly described and illustrated in fifteen species: nine Digenea, *Prosorchis ghanensis*, *Apatemon graciliformis*, *Spirorchis* sp., *Aphalloides coelomicola*, *Proctoeces maculatus*, *Aporocotyle spinosicanalis*, *Schistosoma* sp., *Haematoloechus* sp. and *Echinostoma togoensis*, two Monogenea Polyopisthocotylea, *Heteraxinoides* sp. and *Diplozoon gracile*, two Monogenea Monopisthocotylea, *Cleitharcticus* sp. and *Furnestinia echeneis*, and two Eucestoda, *Moniezia* sp. and *Echeneibothrium* sp. Most of these species have their spermatozoon described for the first time. The literature shows that sperm ultrastructure is now known in about 140 genera (almost 200 species) of parasitic Platyhelminthes (= Aspidogastrea, Digenea, Monogenea, Amphilinidea, Gyrocotylidea, and Eucestoda). The general structure (two 9+"1" axonemes, microtubules only dorsal and ventral in the principal region of the spermatozoon) originates from a process of proximo-distal fusion and is found in the Aspidogastrea, Digenea, Gyrocotylidea and Amphilinidea, with, however, a few variations in the first two groups. The Monogenea Polyopisthocotylea are differentiated by the presence of additional lateral microtubules. The Monogenea Monopisthocotylea are characterised by the loss of certain structures and a general trend towards a simpler spermatozoon. The Eucestoda are characterized by the loss of the mitochondrion and show some other deviations. The genus *Schistosoma*, belonging to the Digenea, has a spermatozoal structure completely different from the general structure found in the two supposedly closely related families of blood-flukes, the Spirorchidae and Sanguinicolidae, and the spermatozoon is considered "progenetic". A precise identification of the outgroup for the parasitic Platyhelminthes is needed for a more reliable cladistic analysis of spermatozoal characters.

RÉSUMÉ

Ultrastructure des spermatozoïdes et phylogénie des Plathelminthes parasites

L'ultrastructure du spermatozoïde et de la spermiogenèse est décrite brièvement et illustrée chez quinze espèces: neuf Digenea, *Prosorchis ghanensis*, *Apatemon graciliformis*, *Spirorchis* sp., *Aphalloides coelomicola*, *Proctoeces maculatus*, *Aporocotyle spinosicanalis*, *Schistosoma* sp., *Haematoloechus* sp. et *Echinostoma togoensis*, deux Monogenea Polyopisthocotylea, *Heteraxinoides* sp. et *Diplozon gracile*, deux Monogenea Monopisthocotylea, *Cleitharcticus* sp. et *Furnestinia echeneis*, et deux Eucestoda, *Moniezia* sp. et *Echeneibothrium* sp. Le spermatozoïde est décrit pour la première fois dans la plupart de ces espèces. Les données bibliographiques montrent que l'ultrastructure du spermatozoïde est maintenant connue chez approximativement 140 genres (presque 200 espèces) de Plathelminthes parasites (= Aspidogastrea, Digenea, Monogenea, Amphilinidea, Gyrocotylidea, et Eucestoda). La structure générale (deux axonèmes 9+"1", microtubules seulement dorsaux et ventraux dans la région principale du spermatozoïde) résulte d'un processus de fusion proximo-distale et est rencontrée chez les Aspidogastrea, Digenea, Gyrocotylidea et Amphilinidea, avec toutefois quelques variations dans les deux premiers groupes. Les Monogenea Polyopisthocotylea se différencient de

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Source : MNHN, Paris

la structure symplésiomorphe par la présence de microtubules latéraux additionnels. Les Monogenea Monopisthocotylea sont différenciés par la perte de nombreuses structures et une tendance générale vers un spermatozoïde plus simple. Les Eucestoda sont caractérisés par l'absence de la mitochondrie et montrent d'autres déviations. Le genre *Schistosoma*, appartenant aux Digenea, a un spermatozoïde à structure totalement différente de la structure générale qui est pourtant retrouvée dans les familles supposées proches (Spirorchidae et Sanguinicolidae), et son spermatozoïde est considéré comme "progénétique". L'outgroup des Platyhelminthes parasites doit être identifié de manière précise pour une analyse cladistique plus fiable des caractères du spermatozoïde.

The phylum Platyhelminthes represents an exemplary case of the use of comparative spermatology for the understanding of phylogeny, because it combines several characteristics: 1. absence of fossils, necessitating that characters may be sought only in living taxa; 2. small size of organisms, which have directed the interest of researchers toward electron microscopy from the onset of this technique; 3. sperm morphologies which, although they follow a general ground plan, are extremely variable; 4. in most parasitic Platyhelminthes, an enormous development of the reproductive apparatus and a constant fecundity, making the work of the electron microscopist easier.

Moreover, some "historical" factors have emphasised the importance of comparative spermatology in the Platyhelminthes. The first is that several schools of research have been created in this field and continue to be investigating actively. Professor EUZET has established, in the seventies, the first of these schools in Montpellier, from which were successively issued the theses of KTARI (1971)[135], MOKHTAR-MAAMOURI (1976)[152], FOURNIER (1980)[60], and JUSTINE (1980) [88], (1985)[95]. The work of SWIDERSKI [58, 209-222] was linked with this school. A second generation of researchers has been formed by the students of EUZET: AZZOUZ-DRAOUI (1985)[6] and NOURY-SRAÏRI (1988)[162]. Xavier MATTEI, commencing with a thesis in the same University of Montpellier on Fish comparative spermatology, went on to establish an active group in Dakar, Senegal, where JUSTINE worked on various Platyhelminthes [88-95, 105-123] and later MARCHAND and BÂ worked on Cestodes [8-21]. Two other independent schools, which are not limited to spermatology but also deal with other ultrastructures, were developed in Australia by Klaus ROHDE (with Nikki WATSON [174, 175, 178, 179, 187-189, 191-193, 233-235, 237-239, 241, 242, 244] and others [140, 141]) and in Germany by Ulrich EHLERS [49-53] (with SOPOTT-EHLERS [202], XYLANDER [250-252], and others). In the past years, several independent teams have begun original works in this field, and the establishment of some researchers in China [40, 65, 142, 143, 199, 255-257] and Korea [2, 82, 83, 201] promises that research on spermatozoa will benefit from the wide diversity of the fauna in these regions.

The second historical factor is that spermatozoal ultrastructure was recognised relatively precociously as a source of characters for the cladistic analysis of the phylum Platyhelminthes. As early as 1984-1985, EHLERS [49-51] and later BROOKS [30] used spermatozoal apomorphies for defining *major* groups of the phylum. The Trepaxonemata Ehlers, 1984 was defined on the basis of an ultrastructural character of spermatozoa (the 9+"1" axoneme). This a striking example of the importance of comparative spermatology for the cladistic analysis of the Platyhelminthes: such a definition would have been impossible ten years before, but it remains unquestioned ten years later. Several groups of Platyhelminthes such as the Temnocephalida [77, 98, 129], the Prolecithophora [53], the Cercomeridea [98] or others [50, 51, 97, 98] have also been defined on the basis of spermatozoal ultrastructural characters.

Table 1 is a list of genera studied for sperm ultrastructure. Comparison with a similar Table published in 1991 [98] shows that the number of genera studied for sperm ultrastructure in the parasitic Platyhelminthes has increased from 98 to 141 (representing almost 200 species) in four years, thus giving an idea of the vitality of these studies in the past years.

This chapter includes new results from several species and a general presentation of sperm ultrastructure in the various taxa of the parasitic Platyhelminthes. The term "parasitic Platyhelminthes" refers in this study to the major taxa (Aspidogastrea, Digenea, Monogenea,

Source : MNHN, Paris

Gyrocotylidea, Amphilinidea and Eucestoda) which include only parasitic species. This study excludes, however, the various parasitic species (see [182]) which belong to the "Turbellaria". The parasitic Platyhelminthes defined above are considered monophyletic in all modern analyses, but are called the Cercomeridea [30] or the Neodermata [181] according to the position assigned to the Udonellidea. Data concerning the "Turbellaria" and Eucestoda are detailed in others chapters of this book by WATSON & ROHDE [232] and BÂ & MARCHAND [21], respectively. The results of the cladistic analyses of the Platyhelminthes previously performed by the author [97-99, 102] will not be repeated here.

MATERIAL AND METHODS

Observations are presented here on the following parasitic species, for which results have not previously been published or have been published in a very incomplete form. Digenea: *Prosorchis ghanensis* Fischtal et Thomas, 1972, Sclerodistomatidae, from the fish *Acanthurus monroviae* Steindachner, 1876, Dakar; *Apatemon graciliformis* Szidat, 1928, Strigeidae, from the bird *Cairina moschata,* Guadeloupe, French West Indies; *Spirorchis* sp., Spirorchidae, from the turtle *Pelusios adansoni*, Lac de Guiers, Senegal; *Aphalloides coelomicola* Dollfus, Chabaud et Golvan, 1957, Cryptogonimidae, from the fish *Gobius micropus*, Mediterranean, France; *Proctoeces maculatus* (Loos, 1901) Odhner, 1911, Fellodistomatidae, from the fish *Crenilabrus cinereus*, Mediterranean, France; *Aporocotyle spinosicanalis* Williams, 1958, Sanguinicolidae, from the fish *Merluccius merluccius* L., 1758, Mediterranean, France; *Echinostoma togoensis* Jourdane et Kulo, 1978, Echinostomatidae, from mice, strain from Togo; *Haematoloechus* sp., Haematoloechidae, from the amphibian *Rana* sp., Dakar, Senegal (spermiogenesis has already been described [111] in this species but one micrograph is added here for comparison); *Schistosoma* sp., Schistosomatidae, from sheep or cattle, Dakar, Senegal (this species has been referred to as *S. bovis* in publications concerning sperm [90, 108] but it has been shown later that certain specimens could belong to *S. curassoni* [3]); Monogenea: *Furnestinia echeneis* (Wagener, 1857), Diplectanidae, from the fish *Sparus aurata* L., Mediterranean, France; *Cleitharcticus* sp., Ancyrocephalidae, from the fish *Acanthurus monroviae* Steindachner, 1876, Dakar; *Heteraxinoides* sp., Heteraxinidae, from the fish *Pomadasys incisus*, Dakar (the spermatozoon has already been described in *Heteraxinoides* sp. from an other fish [123], but not in these specimens); *Diplozoon gracile*, Diplozoidae, from the fish *Gobio gobio*, small river near Montpellier, France (sperm in this species has been described [106, 107] but a micrograph is presented here for comparison); Cestodes: *Moniezia expansa* Rud., 1810, Anoplocephalidae, from sheep, Dakar; cestode, probably *Echeneibothrium* sp., Phyllobothriidae, from the fish *Raja miraletus* L., 1758, Dakar.

Living specimens were placed in cold (4 °C) fixative consisting of 2% glutaraldehyde in a buffer solution of 0.1 M sodium cacodylate, 0.1 M sucrose, and 0.2 mM $CaCl_2$, at pH 7.2 at 4°C for 1 h. After washing in the same buffer, worms were postfixed for 1 h in 1% osmium tetroxide in the same buffer, dehydrated in ethanol and propylene oxide, and embedded in Epon. Ultrathin sections were contrasted with uranyl acetate and lead citrate, and observed with a Siemens Elmiskop 101 or a Hitachi H600 electron microscope.

OBSERVATIONS

Conventions and orientation

Antero-posterior orientation, or "where is the head?" Platyhelminthes spermatozoa are filiform and it is impossible to recognize a "head" or a "tail", in contrast to many other groups. For the orientation of the spermatozoon, some authors have chosen to consider the nucleus as anterior, by analogy to most animal spermatozoa. However, in the Platyhelminthes there is a fundamental antagonism between the orientation of the axonemes and that of the nucleus. In most animal spermatozoa, the anterior extremity of the axoneme, the centriole, pushes the nucleus forward and thus the orientation of the nucleus (considered as anterior in the sperm) and that of the axoneme are the same. In the parasitic Platyhelminthes, the nucleus is located at the non-centriolar extremity of the axonemes. Some limited observations about movement show that the axonemes are motile at the anterior part and that the nucleus is dragged at the posterior part [111]. Observations of fertilization [118, 125] show that the nucleus is the last part of the spermatozoon to enter the oocyte. Therefore, it is more logical to use the antero-posterior orientation in a

Source : MNHN, Paris

TABLE 1. — List of the genera of parasitic Platyhelminthes in which sperm ultrastructure has been studied.

Subclass Trematoda	
Infraclass Aspidobothrea	
Aspidogaster	[22, 71, 72, 197]
Lobatostoma	[182, 183, 193]
Multicotyle	[175, 233, 239]
Rugogaster	[235]
Infraclass Digenea	
Alaria	[75]
Apatemon	[94]
Aphalloides	This study
Aporocotyle	[94, 227-229], This study
Brachylaimus	[254]
Bucephaloides	[57]
Clonorchis	[82, 83, 151]
Collyricloides	[94]
Corrigia	[173]
Cotylophorum	[75]
Cryptocotyle	[171]
Dicrocoelium	[42, 160]
Didymozoon	[115, 116]
Echinostoma	[78, 94], This study
Eurytrema	[63]
Fasciola	[65, 80, 204-206]
Fibricola	[201]
Fischoederius	[143]
Ganeo	[198]
Gonapodasmius	[89, 109, 118]
Gorgodera	[75, 78]
Gynaecotyle	[43]
Haematoloechus	[28, 32-35, 78, 111], This study
Maritrema	[74]
Meiogymnophallus	[48]
Metagonimus	[2]
Metorchis	[142]
Microphallus	[38, 39, 48]
Neochasmus	[47, 81]
Paragonimus	[62, 63, 76, 151, 164, 165, 194, 255]
Pharyngostomoides	[66]
Podocotyle	[64]
Postharmostomum	[40, 143]
Proctoeces	[94], This study
Prosorchis	This study
Pycnoporus	[143]
Quinqueserialis	[249]
Schistosoma	[4, 5, 45, 55, 56, 79, 84, 88, 90, 100, 104, 108, 131, 151, 166-168, 203, 257], This study
Spirorchis	[94], This study
Subclass Cercomeromorphae	
Infraclass Monogenea	
Cohort Polyopisthocotylea	
Atriaster (Atriaster)	[101]
Atriaster (Atrispinum)	[94, 97]
Axine	[120]
Cemocotyle	[123]
Choricotyle	[135, 230]
Concinnocotyla	[244]
Diclidophora	[67, 68, 135, 144, 231]
Diplozoon	[106, 107]
Erpocotyle	[135, 231]
Gastrocotyle	[196]
Gonoplasius	[191]
Gotocotyla	[121, 178]
Heteraxine	[123]
Heteraxinoides	[123], This study
Hexostoma	[119]
Kuhnia	[120]
Lagarocotyle	[73]
Metamicrocotyla	[23]
Microcotyle	[120, 135, 230, 231, 253]
Neopolystoma	[241]
Octomacrum	[69, 70]
Plectanocotyle	[135, 230]
Polylabroides	[192]
Polystoma	[26]
Polystomoides	[174, 177]
Pricea	[244]
Protomicrocotyle	[196]
Pseudodiplorchis	[36]
Pseudomazocraes	[120]
Pterinotrema	[94]
Pyragraphorus	[120]
Sphyranura	[97]
Cohort Monopisthocotylea	
Acanthocotyle	[103, 149, 223]
Amphibdella	[105, 127]
Amphibdelloides	[113]
Anoplodiscus	[234]
Caballerocotyla	[105, 126]
Calceostoma	[105, 124]
Calicotyle	[225, 238]
Cichlidogyrus	[54]
Cleithrarticus	[94, 110], This study
Dionchus	[105, 125, 126]
Diplectanum	[110, 117]
Encotyllabe	[97, 128]
Entobdella	[224, 226]
Euzetrema	[60, 61]
Furnestinia	[105], This study
Gyrodactylus	[134]
Heterocotyle	[110, 112]
Isancistrum	[150]
Lamellodiscus	[105]
Loimosina	[105, 122]
Macrogyrodactylus	[195]
Megalocotyle	[91, 114, 126]
Monocotyle	[238]
Myxinidocotyle	[103, 149]
Pseudodactylogyrus	[139, 151, 195]
Tetraonchoides	[128]
Tetraonchus	[94]
Trochopus	[135, 231]

Source : MNHN, Paris

TABLE 1. — continued.

Infraclass Cestodaria	
Cohort Gyrocotylidea	
Gyrocotyle	[250, 252]
Cohort Cestoidea	
Subcohort Amphilinidea	
Amphilina	[250]
Austramphilina	[182, 187, 188]
Subcohort Eucestoda	
Acanthobothrium	[152, 154, 155, 158, 216]
Aporina	[15]
Avitellina	[14, 18]
Bothrimonus	[145]
Bothriocephalus	[221]
Catenotaenia	[210]
Cotugnia	[14]
Cylindrotaenia	[86, 87]
Diphyllobothrium	[29]
Duthiersia	[96]
Echeneibothrium	[159], This study
Echinobothrium	[6, 7, 58, 156]
Echinococcus	[24, 160, 199]
Glaridacris	[58, 216, 220]
Haplobothrium	[146, 147]
Hymenolepis	[1, 10, 58, 130, 151, 172, 208, 211, 216]
Inermicapsifer	[13, 14, 210, 214]
Lacistorhynchus	[58, 212, 217]
Mathevotaenia	[16]
Moniezia	[9, 209], This study
Monobothrium	[151]
Monoecocestus	[145]
Nematotaenia	[157]
Onchobothrium	[158]
Oochoristica	[222]
Phyllobothrium	[153]
Proteocephalus	[58, 215, 219]
Pseudanthobothrium	[145]
Raillietina	[14, 17, 169]
Retinometra	[12, 218]
Sandonella	[19]
Stilesia	[11]
Taenia	[59]
Tetrabothrius	[207]
Thysaniezia	[20]
Trilocularia	[148]

functional way rather than follow an unjustified analogy with spermatozoa of other groups which have a completely different ontogeny. The nucleus should be considered as *posterior*, and the extremity of the spermatozoon which is thinner, more motile, and devoid of nucleus should be considered *anterior*, because it contains the centrioles.

Dorso-ventral orientation. There are no functional arguments here. The usage follows the purely arbitrary orientation chosen by SATO *et al.* (1967) [194]: mitochondrion ventral, nucleus dorsal. Note that the antero-posterior and dorso-ventral orientations together allow recognition of a right side and a left side for the spermatozoon, a notion important for unilateral organelles such as undulating membranes [121].

Observations on spermiogenesis of some Digenea (Figs 1-3)

Spermiogenesis in Prosorchis *(Fig. 1)*. Spermiogenesis in *Prosorchis* exemplifies the usual process found in most Digenea. The early spermatid, with round nucleus, has a short protuberance (termed zone of differentiation) from which two flagella grow in opposite directions (Fig. 1a). Later, the zone of differentiation elongates (Fig. 1b). The two centrioles are each associated with a striated root (Fig. 1b), and an intercentriolar body is located between them (Fig. 1a). The zone of differentiation has three processes elongating from its distal extremity: a median cytoplasmic process and two free flagella. Transverse sections show that the two flagella have the 9+"1" structure diagnostic of the Trepaxonemata, and that the median cytoplasmic process has dorsal and ventral microtubules. Attachment zones are visible and indicate the place where the flagella will fuse with the median process (Fig. 1c). The process of fusion is proximo-distal, i.e. it begins near the common cytoplasmic mass and ends at the elongating tip of the spermatid. After this fusion, the elongating spermatid (Fig. 1d) shows the same structure as mature spermatozoa: transverse sections show two axoneme, microtubules, and the mitochondrion. In addition, a section of the nucleus is visible in the nuclear region (Fig. 1e).

Source : MNHN, Paris

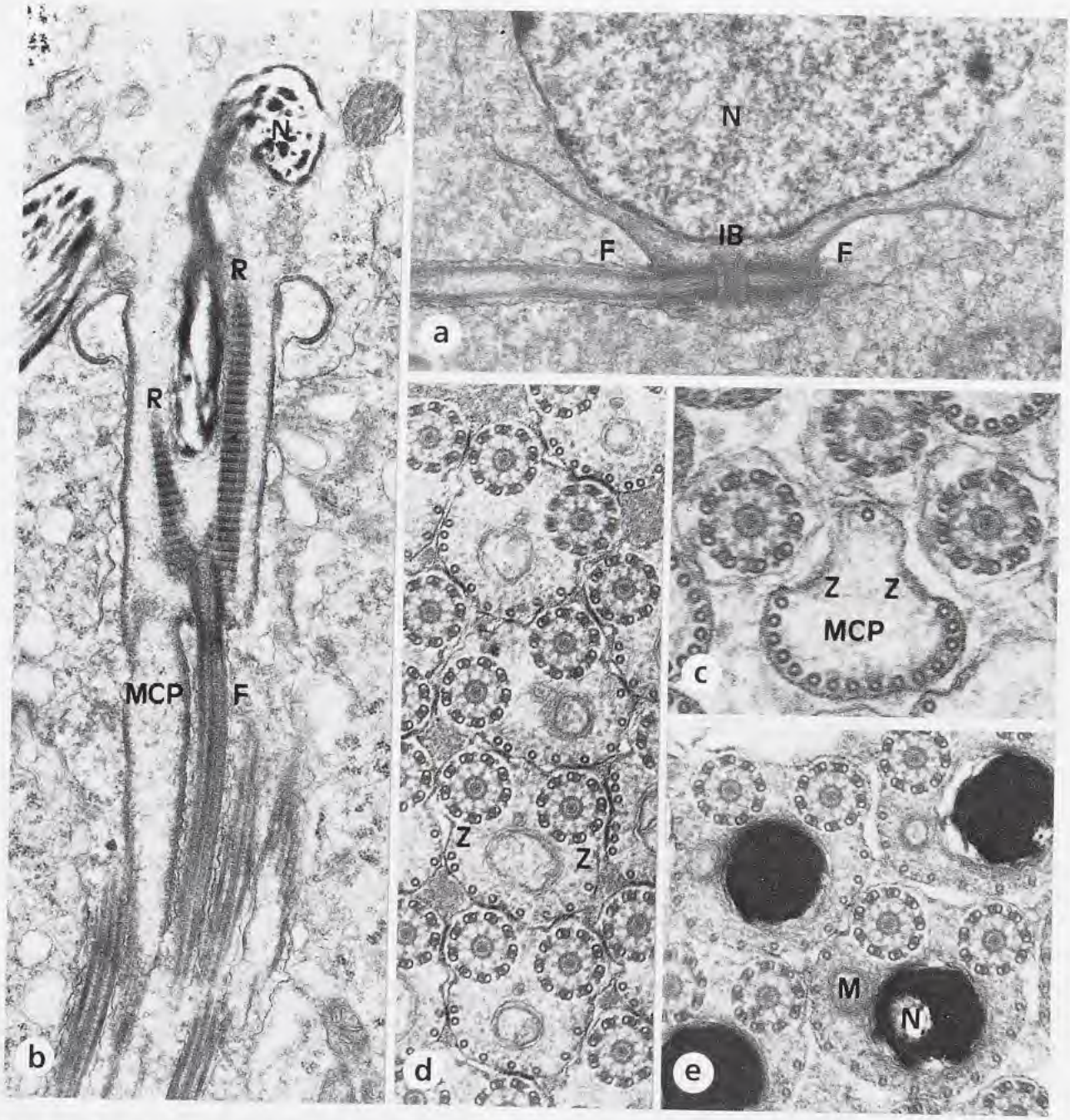

FIG. 1. — Spermiogenesis in the Digenea, exemplified in *Prosorchis ghanensis*. Note the 9+"1" structure of axonemes and microtubules restricted to the dorsal and ventral side of spermatozoa. **a:** Early spermatid, with early zone of differentiation from which two flagella grow in opposite direction. IB, intercentriolar body. **b:** Mature zone of differentiation. The nucleus passes through the zone of differentiation, which bears three elongating processes: the median cytoplasmic process (MCP) and two flagella (one visible here). Each centriole has a striated root (R). **c:** Transverse section in median cytoplasmic process (MCP) and associated flagella. Note attachment zones (Z) where the flagella will later fuse with the MCP. **d:** Transverse section of elongating spermatids, after the fusion; attachment zones (Z) still visible. **e:** Transverse section of elongating spermatid, showing nucleus and mitochondrion. a, x 20 000; b, d, e, x 24 000; c, x 90 000.
For all figures: F, flagellum; M, mitochondrion; N, nucleus.

Source : MNHN, Paris

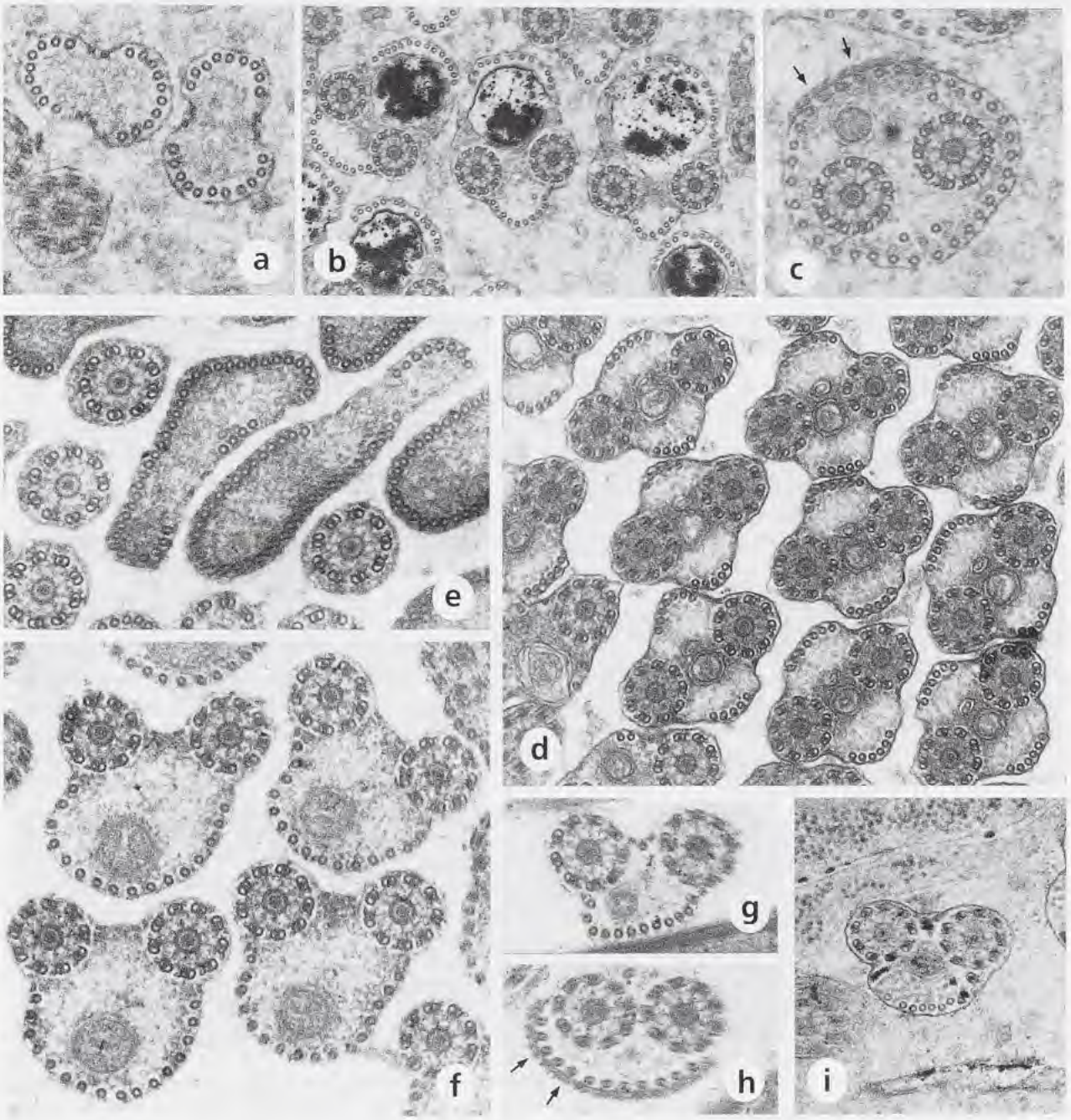

FIG. 2. — Homogeneity of spermiogenesis and spermatozoon structure in the Digenea. **a-c:** *Proctoeces maculatus*. **d:** *Haematoloechus* sp. **e, f:** *Apatemon graciliformis*. **g, h:** *Aphalloides coelomicola*. **i:** *Echinostoma togoensis*. **a, e:** Median cytoplasmic process of spermatid. **d, f, g, i:** Transverse section of spermatozoa in the mitochondrial region. **b:** Transverse section of spermatozoa in the principal region, containing the nucleus. **c, h:** Transverse section in a region (probably anterior) showing ornamentation (arrows) on the membrane. a, c-h, x 60 000; b, x 36 000; i, x 48 000.

Source : MNHN, Paris

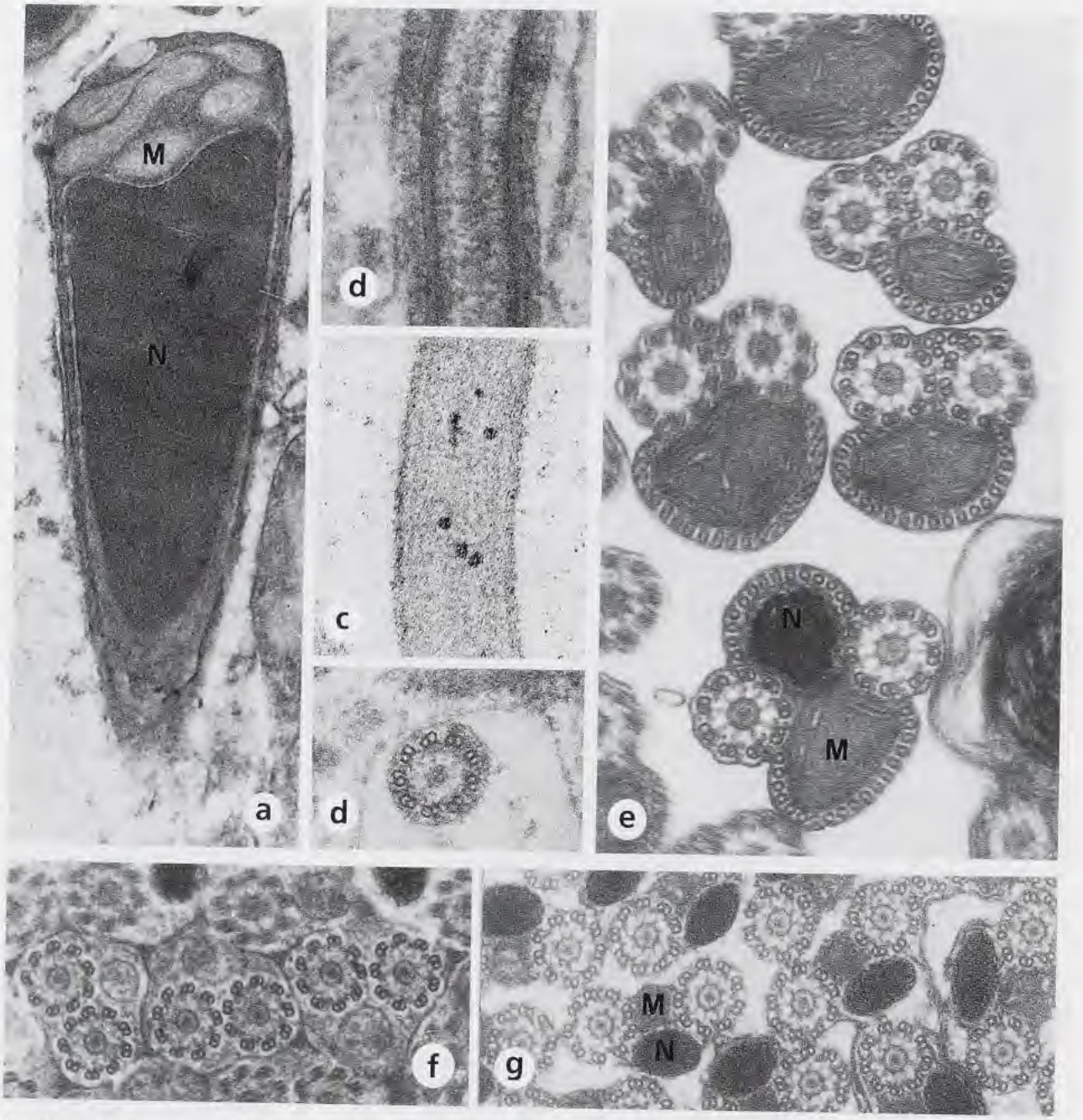

FIG. 3. — Spermatozoa in Schistosomes and other blood flukes. **a-d:** *Schistosoma* sp. (Family Schistosomatidae, blood flukes of mammals). **a:** longitudinal section of sperm body, with mitochondria at the anterior extremity and pyriform nucleus. **b:** longitudinal section of flagella. **c:** glycogen demonstrated in flagella by Thiéry's method. **d:** Transverse section of flagellum, showing a structure which is not a trepaxonematan 9+"1". **e:** *Spirorchis* sp. (Family Spirorchidae, blood flukes of turtles). Transverse section at various levels. **f, g:** *Aporocotyle spinosicanalis* (Family Sanguinicolidae, blood fluke of fishes). Transverse section at the mitochondrial level (f) and nuclear level (g). The spermatozoon of schistosomes is not threadlike and is similar to the zone of differentiation found in spermatids of the other Digenea ("progenetic spermiogenesis"[100]). In contrast, the spermatozoon of the other blood flukes is threadlike and similar to that of other Digenea in having two 9+"1" trepaxonematan axonemes. b-d, modified from [108]. a, x 24 000; b, c, e, x 90 000. d, f, g, x 60 000.

Source : MNHN, Paris

Spermiogenesis in Proctoeces *(Fig. 2 a-c).* The median cytoplasmic process (Fig. 2 a) has a similar as in *Prosorchis*, with its attachment zones clearly visible. Transverse section of spermatozoa in the testis show the usual structure with two axonemes, microtubules, mitochondrion and nucleus. As usual in the Digenea, the microtubules are only dorsal and ventral, and the lateral faces, along the axonemes, are devoid of microtubules (Fig. 2b). However, some transverse section (Fig. 2c) show a continuous microtubule row at the periphery of the spermatozoon. These sections also show external ornamentation associated with the membrane. These sections correspond with the anterior extremity of the spermatozoon, which originates from the zone of differentiation.

Spermatozoon of Haematolechus *(Fig. 2d).* This genus, which is easily collected from frogs, has been widely used for the early studies of spermiogenesis in the Digenea (see Table 1) and has become a standard. Sections of spermatozoa are displayed here to show the similarities of the other species with this standard. Sections show the mitochondrion and a small circular profile of two membranes, which probably represents the extreme anterior extremity of the nucleus.

Spermiogenesis in Apatemon *(Fig. 2e, f).* The median cytoplasmic process (Fig. 2e) shows the usual structure, but the number of microtubules is relatively high. Transverse sections of spermatid after the fusion still show the attachment zones (Fig. 2f).

Spermatozoa of Aphalloides *(Fig. 2g, h) and* Echinostoma *(Fig. 2i).* Spermatozoa show the usual structure, including a zone with external ornamentation in *Aphalloides* (Fig. 2h).

*Spermatozoa in Schistosomes (mammal blood-flukes) and other blood-flukes: Sanguinicolidae or fish blood-flukes (*Aporocotyle*) and Spirorchidae of turtle blood-flukes (*Spirorchis*) (Fig. 3).* Schistosomes show an aberrant sperm structure [90, 100, 104, 131]. The spermatozoon is not filiform (Fig. 3a), and there is one single axoneme, which has not the 9+"1" trepaxonematan structure. Instead, the axoneme shows 9 doublets and a central poorly contrasted structure, termed 9+0 or 9+"1" [108], and is devoid of dynein arms (Fig. 3 b-d). The phyletic interpretation of this aberrant structure is difficult and requires a study of the families considered as close to the schistosomes, i.e. the other blood-flukes, the spirorchid and sanguinicolids. In the spirorchid *Spirorchis*, the spermatozoon (Fig. 3e) is filiform and shows the usual structure found in the Digenea, with two 9+"1" axonemes and dorso-ventral microtubules. In the sanguinicolid *Aporocotyle*, the spermatozoon (Fig. 3 f-g) is filiform and shows two 9+"1" axonemes, but the peripheral microtubules are absent in most sections, although a few microtubules can be seen in some rare sections.

Observations on spermatozoa of the Monogenea Polyopisthocotylea (Figs 4-6)

Spermatozoon of Heteraxinoides *(Fig. 4).* Observations are briefly reported to exemplify the homogeneous structure found in most polyopisthocotylean monogeneans. The spermatozoon shows two 9+"1" axonemes, the nucleus and mitochondrion (Fig. 4). The fundamental difference from the Digenea is that the microtubule row, in the principal region of the spermatozoon, makes a complete circle around the sperm cell, i.e. it is not interrupted at the axoneme level.

Spermatozoon of Diplozoon *(Fig. 5).* The spermatozoon is highly aberrant [105-107]: it is elongate, aflagellate and contains several hundreds of parallel longitudinal microtubules (Fig. 5b). A view of the animal is presented (Fig. 6a) for the purpose of the discussion.

Spermatozoon of Gotocotyla *(Fig. 6).* This species has the general pattern found in the polyopisthocotylean monogeneans but shows an outstanding additional structure: a lateral undulating membrane, which contains a large number of parallel microtubules. The ultrastructure of the spermatozoon has been described previously [121], but Fig. 6 is an unpublished artist's view of the mature spermatozoon drawn from micrographs.

Source : MNHN, Paris

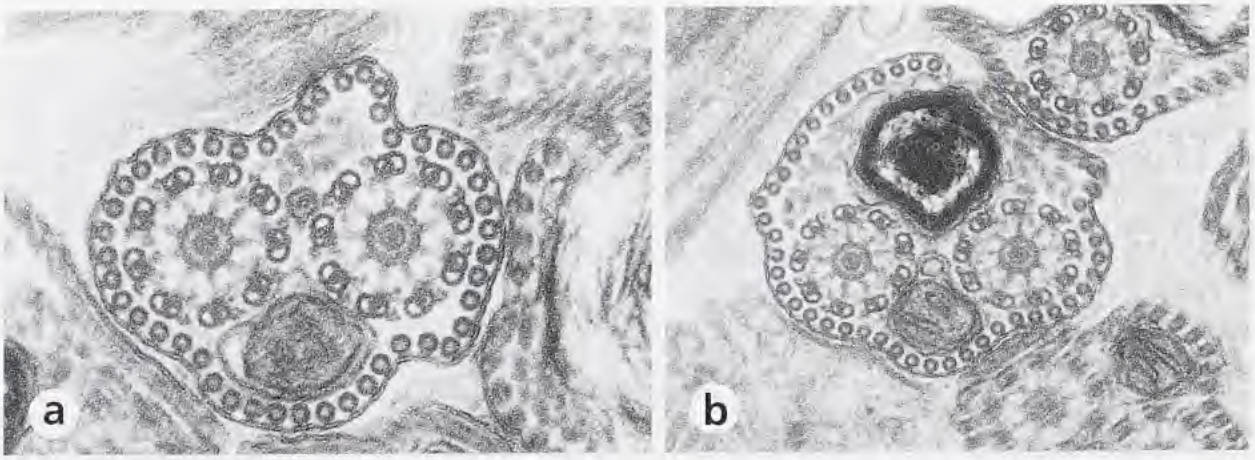

FIG. 4. — Spermatozoa in the Monogenea Polyopisthocotylea, exemplified by *Heteraxinoides* sp. **a, b:** Transverse sections showing two 9+"1" axonemes, mitochondrion and nucleus. Note that the microtubules form a complete row around the spermatozoon (synapomorphy for the Polyopisthocotylea [97, 98]). a, x 90 000; b, x 120 000.

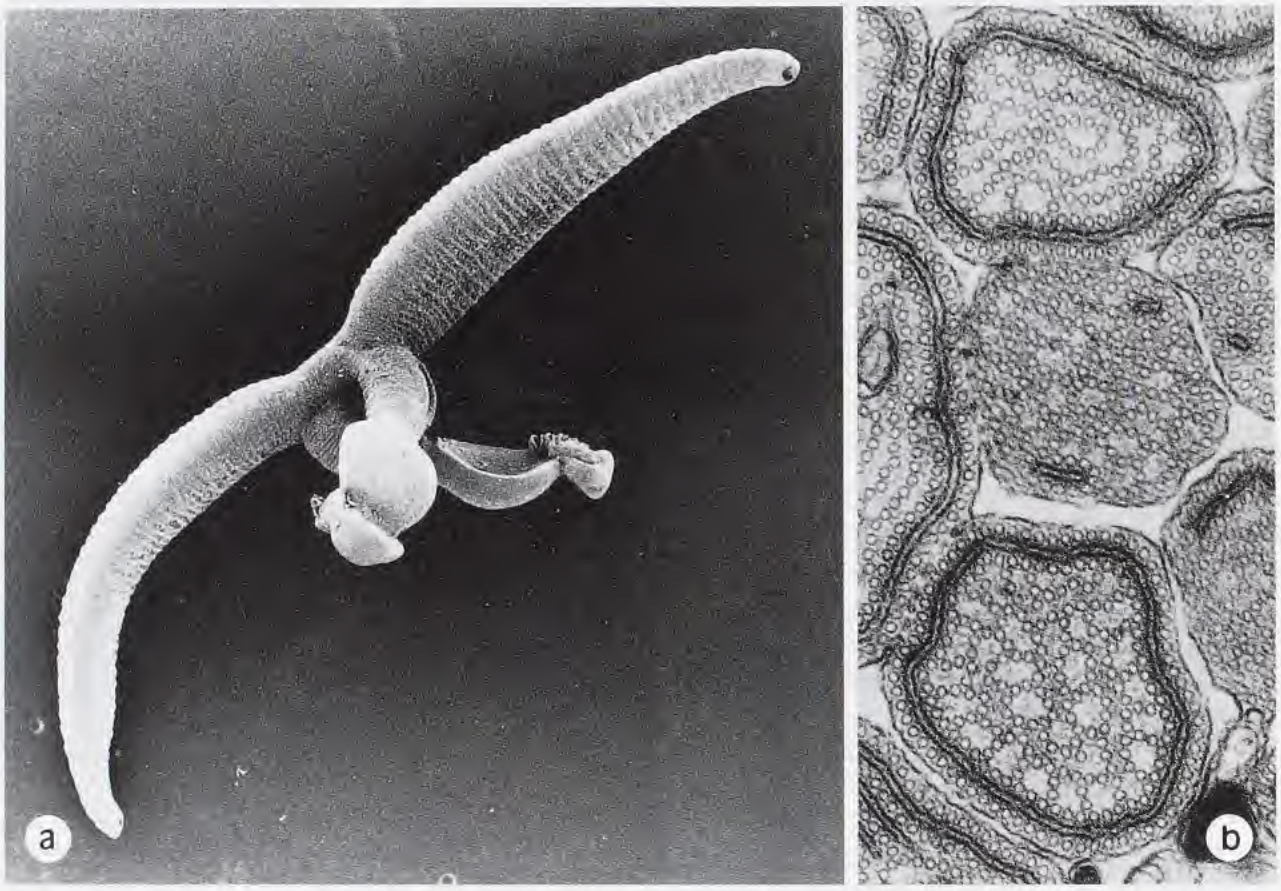

Fig. 5. — The aberrant case of *Diplozoon* (Monogenea Polyopisthocotylea). **a:** The two member of a pair are permanently fused. Scanning electron microscope photograph of *Diplozoon nipponicum* by Nathalie LE BRUN. **b:** Transverse section of spermatozoa of *Diplozoon gracile*, showing numerous parallel longitudinal microtubules (modified from [106]). This is the only known case of aflagellate spermatozoon in the parasitic Platyhelminthes, and this aberrant pattern is linked with the exceptional biology of reproduction.

Source : MNHN, Paris

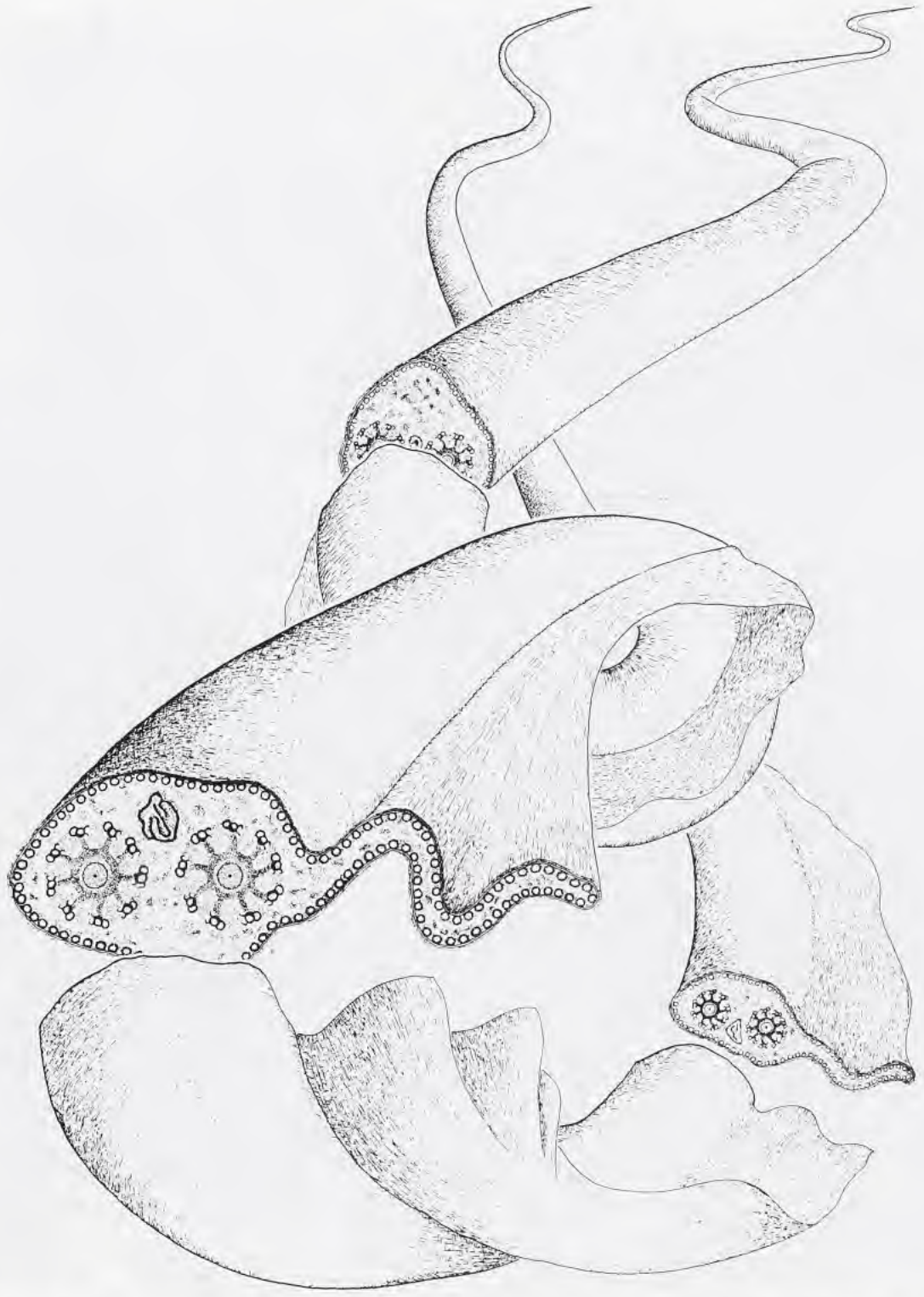

FIG. 6. — An artist's view of the spermatozoon of *Gotocotyla acanthura* (Monogenea Polyopisthocotylea), drawn from micrographs published in [121]. The spermatozoon has an undulating membrane along part of its length. The undulating membrane is an autapomorphy for this species. However, the general sperm structure is similar to the classic pattern found in the parasitic Platyhelminthes. Drawing by Nathalie LE BRUN.

Source : MNHN, Paris

Observations on spermiogenesis of the Monogenea Monopisthocotylea (Figs 7, 8)

The Monopisthocotylea show a variety of sperm structures. Observations reported here concern two species in which the spermatozoon has one single axoneme, and thus has the maximum deviation from the Digenea or the Polyopisthocotylea.

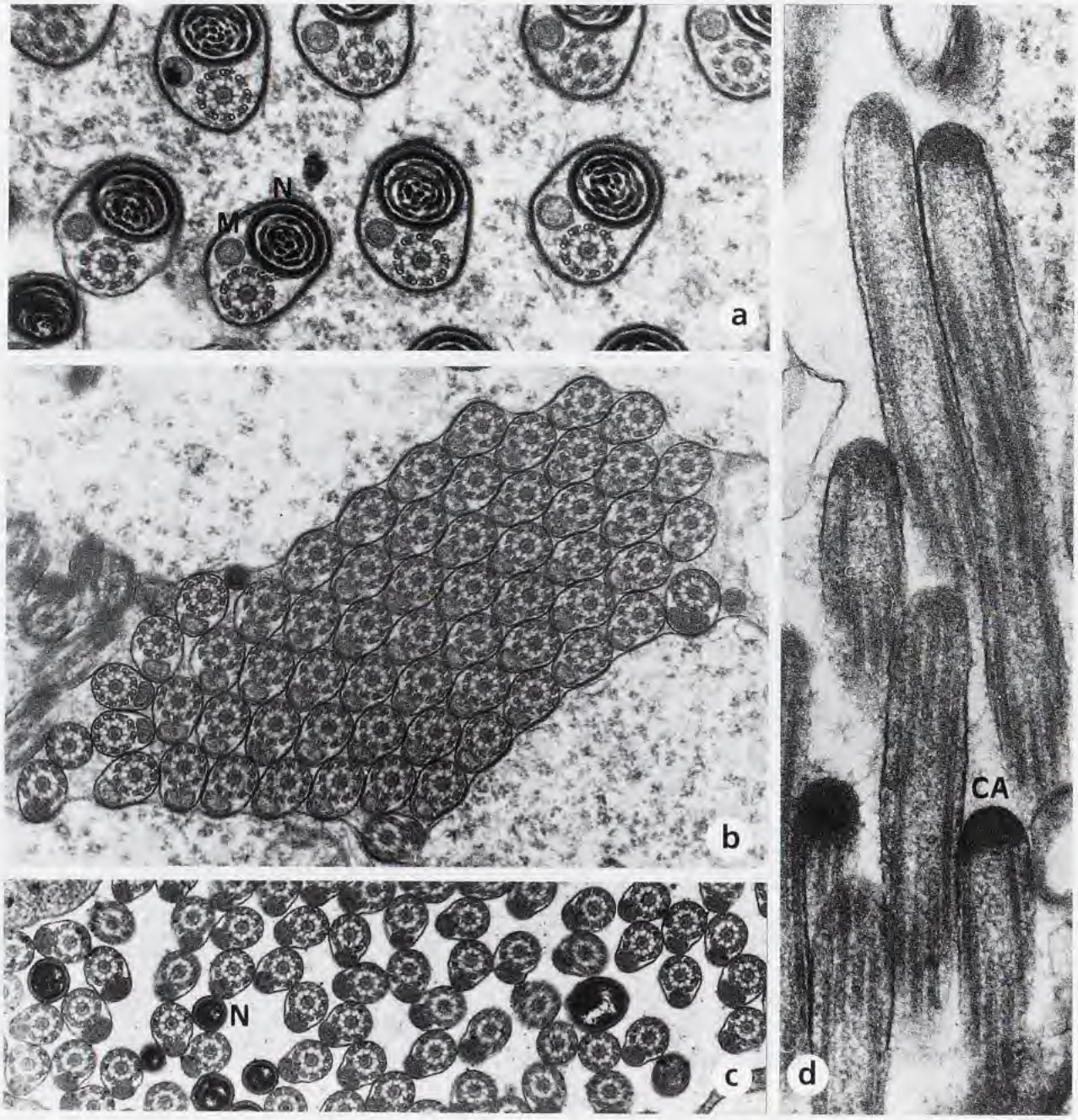

FIG. 7. — Spermiogenesis in *Furnestinia echeneis*, a Monogenea Monopisthocotylea with uniflagellate spermatozoon. **a:** Transverse section of zones of differentiation embedded in the common cytoplasmic mass (modified from [105]). **b:** Isogenic group with 64 spermatids; **c:** Mature spermatozoa have only one axoneme, and the nucleus is located in a region devoid of any other organelles. **d:** centriolar adjunct. a, d, x 48 000; b, c, 30 000.

Source : MNHN, Paris

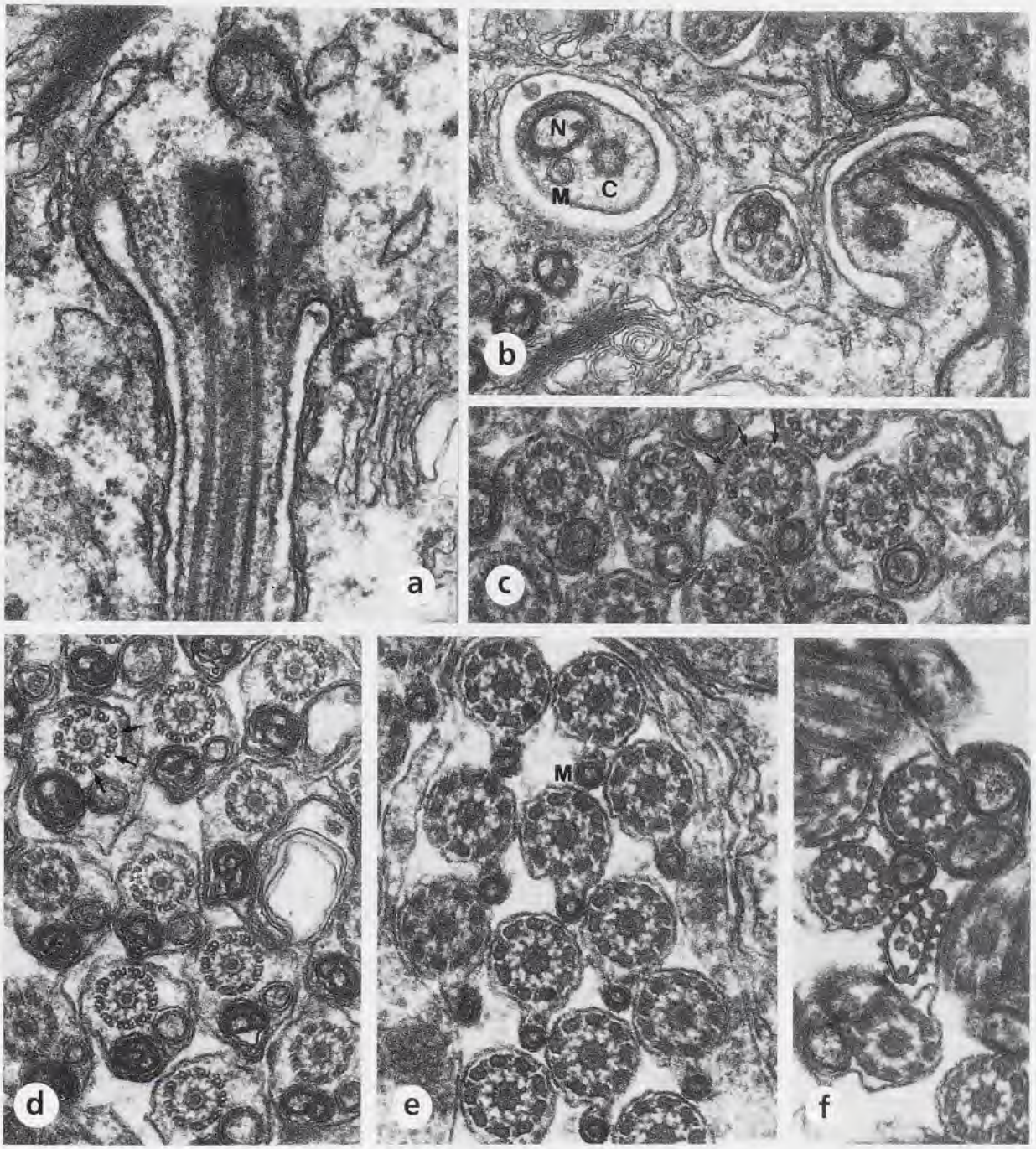

FIG. 8. — Spermiogenesis in *Cleitharcticus* sp., a Monogenea Monopisthocotylea with uniflagellate spermatozoon. **a:** Longitudinal section of zone of differentiation containing one single axoneme. Note absence of intercentriolar body and striated roots (compare with Fig. 1a, b). **b:** Transverse section of zones of differentiation, showing single centriole and migrating nucleus and mitochondrion. **c, d:** Axonemes in growing spermatids show an incomplete tubule b (arrows) in the axoneme. **e:** Mature spermatozoa, however, have complete tubules b in their axoneme. **f:** Region with external ornamentation located at one extremity of the spermatozoon. a, d, x 48 000; b, x 30 000; c, e, f, x 60 000.

Source : MNHN, Paris

Spermiogenesis in Furnestinia *(Monopisthocotylea) (Fig. 7).* During spermiogenesis, the zone of differentiation is deeply embedded in the common cytoplasmic mass, and shows one single axoneme, the migrating nucleus and mitochondrion (Fig. 7a). It is not possible to recognize the proximo-distal fusion in this species, since only one element exists from the beginning to the end of spermiogenesis. Isogenic groups of spermatids comprise 64 elements (Fig. 7b). Mature spermatozoa in genital ducts show a relatively simple structure, with a region showing the axoneme and mitochondrion, and an other region showing the nucleus with no accompanying element (Fig. 7c). The anterior extremity contains a centriolar derivative (Fig. 7c). Cortical microtubules are absent at all stages of spermiogenesis.

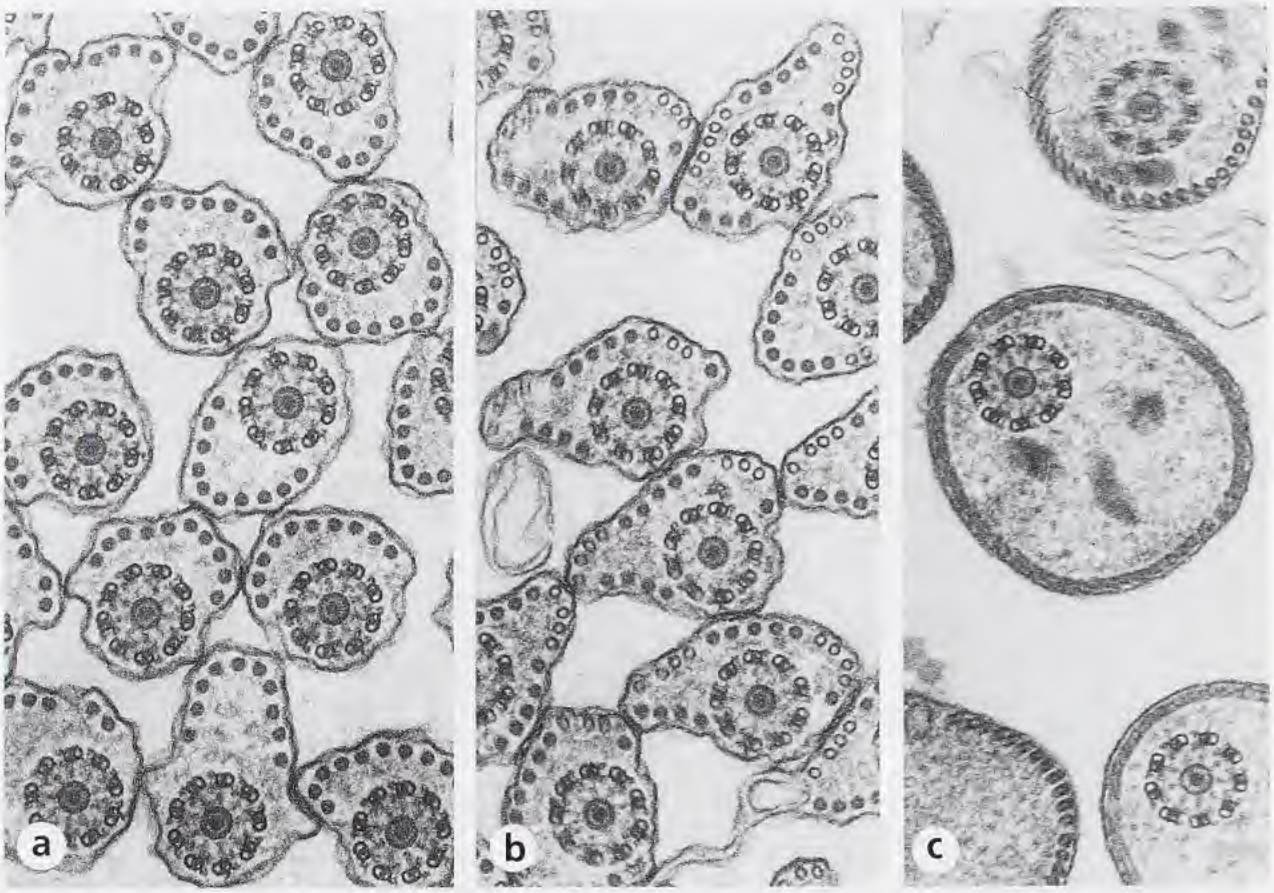

FIG. 9. — Spermatozoa of Eucestoda. Note the absence of mitochondrion, a synapomorphy for the Eucestoda. **a, b:** *Echeneibothrium* sp. Microtubules and axonemes are at focus on the same section. Note presence of two kinds of microtubules, with thin wall and thick wall. **c:** *Moniezia* sp. Note that the axoneme and the peripheral microtubules are not in perfect cross section on the same sections, because of the peripheral microtubules twisting, a synapomorphy for the Cyclophyllidea [98]. a-c, x 60 000.

Spermiogenesis in Cleitharcticus *(Monopisthocotylea) (Fig. 8).* The zone of differentiation (Fig. 8a) is deeply embedded in the cytoplasmic mass and shows one single centriole; the intercentriolar body and striated roots found in the Digenea are absent. As for *Furnestinia*, it is not possible to recognize the proximo-distal fusion in this species. In maturing spermatids, the axoneme shows incomplete b tubules (Fig. 8c, d), but these are complete in the mature sperm

Source : MNHN, Paris

cells (Fig. 8e, f). Mature sperm cells show one single axoneme, the nucleus and mitochondrion, and are devoid of cortical microtubules (Fig. 8f). Some rare sections (Fig. 8f) show external ornamentation on the membrane and may, with analogy with the Digenea, be considered as anterior, although this has not been fully demonstrated.

Observations on spermatozoa of the Eucestoda (Fig. 9)

A few observations are reported here to demonstrate the major characters of spermatozoa. The two species shown have a single axoneme, but others have two axonemes.

Spermatozoa of Echeneibothrium *(Fig. 9a, b).* Transverse sections show one single axoneme of the 9+"1" structure. The nucleus, not cut at the levels shown here, is present in other sections. Sections never show a mitochondrion. An interesting feature is the co-existence of two kinds of microtubules, either with thin wall and thick wall.

Spermatozoa of Moniezia *(Fig. 9c).* The spermatozoon has a single axoneme and is devoid of mitochondrion. Transverse sections of sperm never show both the peripheral microtubules and the axoneme perfectly transverse because the microtubules are twisted around the sperm body.

DISCUSSION

Structure of the spermatozoon in the major groups of parasitic Platyhelminthes

The basic structure: Digenea, Amphilinidea, Gyrocotylidea. The basic structure is here described for the Digenea but appears to be similar in the Amphilinidea and Gyrocotylidea. This structure is a synapomorphy for the Cercomeridea [98]. However, within the Cercomeridea, other structures have evolved from this basic pattern, and this pattern may be considered the symplesiomorphic structure for the parasitic Platyhelminthes.

The spermatozoon is filiform, very long, and therefore can be described only from transverse sections. It is composed of two main regions: the principal region, posterior, originating from the fusion of the three processes attached to the zone of differentiation of the spermatid; and the anterior region, originating from the zone of differentiation itself [98]. Transverse sections of the spermatozoon in the principal region (which contains the nucleus) show two axonemes, the mitochondrion and nucleus, and peripheral longitudinal microtubules limited to the ventral and dorsal faces. The microtubules are not twisted along the long axis of the sperm and are parallel. The anterior region is much shorter than the principal region. It generally shows a continuous microtubule row, not interrupted at the axoneme level, and is often marked by external ornamentations on the membrane.

It should be emphasized that, although the structure found in the principal region is remarkably homogeneous, a greater variety of structure is found in the anterior region. The anterior region of *Proctoeces* (Fig. 2c) has the structure described above, but *Aphalloides* (Fig. 2h) has ornamentation in a region which lacks a complete row of microtubules. In *Haematoloechus*, the two regions (anterior and principal) are separated by a "collerette" or collar which is visible with the light microscope [111]. The variations of the anterior region and its associated membrane ornamentation would probably be valuable for the understanding of phylogeny, because the principal region is too homogeneous to be useful. However, transverse sections of the anterior region often represent only a small proportion of the sections available, and sub-optimal fixations do not allow observation of the membrane ornamentation.

Variation in the Digenea. Sperm structure in the Digenea is homogeneous and variations are relatively rare.

In the didymozoids, deviations from the classic pattern have been found in *Gonapodasmius* and *Didymozoon*. *Gonapodasmius* has a relatively short spermatozoon, and the intercentriolar

Source : MNHN, Paris

body is lacking during spermiogenesis, but the ultrastructure of the spermatozoon is "classical" and the morphology is filiform. *Didymozoon* has a filiform spermatozoon, but the two axonemes show a 9+0 pattern and peripheral microtubules are absent.

In the Schistosomes (genus *Schistosoma*) the deviation from the symplesiomorphic pattern is extreme (Table 2).

TABLE 2. — Characteristics of spermiogenesis in the schistosomes and other Digenea (See Table 1 for references)

Characteristic	Digenea	*Schistosoma*
Shape of spermatid	great elongation, spermatozoon filiform	no elongation
Centrioles	migration from distal extremity of zone of differentiation to proximal extremity	no migration
Median cytoplasmic process	lengthening	abortion of process growth
Free flagella	lengthening, up to 400 µm	moderate lengthening (20 µm)
Shape of nucleus	successively round, pyriform and eventually very long and filiform	successively round and pyriform; no further elongation
Migration of nucleus	nucleus migrates to a region posterior to the centriolar region	no migration; nucleus remains in a position anterior to the centriolar region
Shape of mitochondria	mitochondria gather to fuse and form a single filiform and much elongated mitochondrion	mitochondria remain ovoid and do not fuse
Migration of mitochondria	the single mitochondrion migrates to a region posterior to the centriolar region	mitochondria remain anterior to centriolar region (and to nucleus)
Evolution of striated roots	striated roots, originally conspicuous, disappear at the end of spermiogenesis	striated roots remain in mature spermatozoa
Centriole ultrastructure	originally 9 triplets in spermatids, simplifies to 9 singlets	9 triplets in spermatid and in spermatozoon
Axoneme structure	in early spermatid, axonemes are sometimes observed without trepaxonematan core (9+0 structure); trepaxonematan 9+"1" in all other stages including spermatozoon	central trepaxonematan core absent in all stages
Separation of germ cells	spermatocytes form clusters; spermatids remain grouped in a common cytoplasmic mass until the very end of spermiogenesis	germ cells separate early in spermatogenesis; no clusters, no cytoplasmic mass

Source : MNHN, Paris

Sperm structure in the schistosomes and its deviation from the classic pattern is difficult to interpret if only a comparison between mature spermatozoa is performed. However, a comparison of the spermiogenetic processes shows that the mature sperm of schistosomes can be compared to a mature zone of differentiation in other Digenea. Spermiogenesis in the schistosomes therefore may be considered "progenetic" (i.e. the mature spermatozoon is a spermatid's zone of differentiation having precociously reached maturity) [100]. In addition, this zone of differentiation has one single axoneme instead of two.

It is interesting to note that the deviation in sperm pattern found in *Schistosoma* is not found in the other blood-flukes, the spirorchids and the sanguinicolids. These families, however, are considered to be closely related to the schistosomes in most phyletic schemes of the Digenea [31, 37, 44, 136, 200], but a different opinion was recently expressed [25]. The schistosomal sperm pattern is therefore restricted to the family Schistosomatidae. Moreover, in the genus *Schistosomatium*, a member of the family Schistosomatidae, light microscope observations have shown that there are clusters and that the spermatozoon is filiform, with two free flagella at one extremity [161]. Thus, the schistosome aberrant pattern appears to be restricted to the genus *Schistosoma* but is similar in all species of the genus [104].

The link between phylogeny and sperm ultrastructure is thus not obvious for the schistosomatids and related families (spirorchids, sanguinicolids). The influence of the biology of fertilization is probably important, and is discussed below.

Sperm ultrastructure in the Aspidogastrea. The Aspidogastrea are considered the most primitive group of Neodermata by ROHDE [183]. Indeed, their spermatozoa correspond to the classic, symplesiomorphic pattern, with two 9+"1" axonemes and dorso-ventral microtubules. However, some deviations from the plesiomorphic pattern do exist in the Aspidogastrea and are listed in Table 3 for three species; information available on *Aspidogaster* is scarce and therefore not included. The dense region could be considered a synapomorphy for the Aspidogastrea [235]. The undulating membrane of *Lobatostoma* [176, 193] and *Multicotyle* [239] is similar to that of the monogenean *Gotocotyla* [121] and should be considered a case of convergence.

TABLE 3.— Deviation from the basic pattern of the Cercomeridea found in the Aspidogastrea.

Characteristic	*Multicotyle*	*Lobatostoma*	*Rugogaster*
Dense region	Present	Present	Present in spermatid, not seen in mature sperm
Undulating membrane	Present	Present	Absent (but see Fig. 27)
Reference	[233, 239]	[193]	[235]

Spermatozoon structure in the Monogenea Polyopisthocotylea. The deviation from the symplesiomorphic pattern in the Polyopisthocotylea is the acquisition of supplementary microtubules on the lateral faces of the mature spermatozoon. This has been proposed as a synapomorphy for this group [97]. The Polyopisthocotylea are relatively homogeneous in their sperm structure, but some original features have been found in a few cases. These are generally restricted to one species and thus should be considered only as autapomorphies [101]. *Diplozoon*

Source : MNHN, Paris

has an aberrant spermatozoon with more than 400 parallel longitudinal microtubules and no axonemes. This represents the only case of aflagellate sperm in the parasitic Platyhelminthes, but aflagellarity has also evolved several times in the "Turbellaria" ([163, 190, 202]; see also [232]). The case of *Diplozoon* is dealt with below, in the paragraph on biology of fertilization.

Sperm structure in the Monogenea Monopisthocotylea. The Monopisthocotylea are characterised by synapomorphies such as the loss of the dorso-ventral microtubules, loss of striated roots, loss of intercentriolar body. A general evolutionary trend in this group is a tendency to shorter and simpler spermatozoa. Simplification of sperm includes the loss of one axoneme and the loss of peripheral microtubules, with the result that the sperm cell contains only three components, the nucleus, mitochondrion and one axoneme. A general cladistic analysis of sperm structure has been performed on the Monopisthocotylea [97] and updated [102]. These analyses, among other results, have allowed the erection of the group Monoaxonematidea Justine, 1991 (etymology: one single axoneme) for several families which have uniflagellate spermatozoa [97]. Since new information has been acquired recently on several Monogenea (see Table 1), this cladistic analysis should be updated. This would, however, exceed the scope of this general review.

The recent literature shows that DNA studies generally accord with spermatology concerning the systematic relationships within the Monogenea. JUSTINE (1991) [97, 98] insisted that synapomorphies can be defined for the Monopisthocotylea and for the Polyopisthocotylea, but not for the Monogenea as a whole (= Monopisthocotylea+Polyopisthocotylea). Subsequently, several DNA studies have also concluded that no argument could be given to support monophyly of the Monogenea [27, 46, 183-185]. The position of the Gyrodactylids within the Monopisthocotylea or the Polyopisthocotylea is uncertain in studies based on morphology [137] but they are clearly assigned to the Monopisthocotylea on spermatological evidence [97, 105]; this has been recently confirmed in a study of 18S DNA [46].

The convergence found between the Monopisthocotylea and the Eucestoda has received little comment. Both groups, and these groups alone, within the parasitic Platyhelminthes, have filiform spermatozoa with one single axoneme. This emphasizes a striking feature of the Platyhelminthes: the plesiomorphic sperm in the parasitic group is biflagellate, and these two groups have evolved toward a simpler structure.

Sperm structure in the Eucestoda. The Eucestoda are characterized by a synapomorphy accepted by all authors [21, 30, 50], the absence of mitochondrion. This clearly apomorphic condition separates the Eucestoda from the two other groups of the Cestodaria, the Amphilinidea and Gyrocotylidea. These two groups have apparently kept the basic structure of the Cercomeridea. This character-state "absence of mitochondrion" requires some comments. Characters defined by "absence" are generally considered, in systematics and particularly in cladistics, as less reliable than the acquisition of new structures. However, in this case, one may note that the absence of mitochondrion is probably correlated with the acquisition of other enzymatic systems in the spermatozoon, which are not revealed by ultrastructural methods. Another comment is that researchers interested in the inheritance of paternal mitochondrial DNA should perform a comparative study between a digenean, in which the volume of the spermatozoal mitochondrion is important, and a member of the Eucestoda.

Other synapomorphies of Eucestoda spermatozoa include the twisting of peripheral microtubules, a synapomorphy for the Cyclophyllidea [98] exemplified in the present paper. BÂ & MARCHAND have detailed propositions of other synapomorphies in their chapter in this volume [21].

Source : MNHN, Paris

Number of spermatids in an isogenic group (32 or 64), and value of this character for phylogenetic studies

Table 4 gives a list of some references concerning the number of spermatids in isogenic groups, i.e. groups of cells originating from a single spermatogonium, and fused together until the separation of the mature spermatozoa. XYLANDER [250] has given some phylogenetic value to this character, and considered that a group of 64 was a synapomorphy for the Amphilinidea+Gyrocotylidea+Eucestoda. However, some problems make this character difficult to use. The number is not homogeneous in the Monogenea Monopisthocotylea nor in the Aspidogastrea. Also, it is difficult to polarise this character since the outgroup for the parasitic Platyhelminthes is not precisely known. This character cannot be used at the present time owing to these uncertainties.

TABLE 4. — Number of spermatids in an isogenic group

Group	Species	Number of spermatids	Reference
Aspidogastrea			
	Rugogaster	64	[235]
	Multicotyle	32	[233, 239]
Digenea			
		32	many, see Table 1
Monogenea Polyopisthocotylea			
	Diclidophora	32	[67]
	Gastrocotyle	64	[196]
	Gonoplasius	64	[191]
	Protomicrocotyle	64	[196]
	Several (see Table 1)	64	[135]
Monogenea Monopisthocotylea			
	Calceostoma	32	[124]
	Calicotyle	64	[238]
	Diplectanum	64	[117]
	Macrogyrodactylus	32	[195]
	Megalocotyle	64	[114]
	Myxinidocotyle	64	[103]
	Pseudodactylogyrus	32	[195]
	Pseudodiplorchis	32	[36]
Gyrocotylidea			
	Gyrocotyle	64	[250]
Amphilinidea			
	Amphilina	64	[250]
Eucestoda			
	many	64	Table 1, [21]

Source : MNHN, Paris

Organelles absent in parasitic Platyhelminthes but present in free-living Platyhelminthes ("Turbellaria"): dense bodies and 25 nm granules

As for other characters mentioned above, the apomorphic character of the parasitic Platyhelminthes is the absence of structures which are present in the plesiomorphic taxa.

Membrane-bound dense bodies. These are absent in all parasitic Platyhelminthes examined. Several categories of granules are present in the same spermatozoon in the Acoela [170]. In other taxa of the "Turbellaria", one or two categories are found, or dense bodies are absent (see Table 1 in [232]). The absence of membrane-bound granules has been considered a synapomorphy for the Neodermata by EHLERS [50, 51].

Cortical non-membrane bound 25-nm granules. These have been found in the Temnocephalidea [77, 180, 240, 243, 245, 246] and in some Dalyellioida [41, 163, 202]. Detailed bibliographical information can be found in the chapter by WATSON & ROHDE in this volume [232]. These granules have been discussed by several authors [77, 163, 202, 232]. It is pertinent to this chapter that they have never been found in any parasitic Platyhelminthes. In our present state of knowledge, it does not appear valid to attribute to the presence of these granules the value of a synapomorphy for a specific taxon of the "Turbellaria" because their presence is not regular in all taxa of a given group. However, they could have an important physiological role, which apparently does not exist in any parasitic Platyhelminthes.

Spermatozoal structure and the biology of fertilization

The question of the relationships between spermatozoal structure and the biology of fertilization in Platyhelminthes has been discussed before [98] but new data make it more precise. It is impossible to link the aberrant structure found in *Schistosoma* (Digenea) and in *Diplozoon* (Monogenea) with the assumed respective phylogeny of these two taxa. For the schistosomes, the present study shows that related families have the plesiomorphic structure. For *Diplozoon*, a recent study [70] has shown that the Octomacridae, considered very close to the Diplozoidae, have the symplesiomorphic structure. All spermatologists know that the biology of fertilization may affect the morphology of spermatozoa: in a taxonomic group, the species which evolve toward a different biology of fertilization may be expected to acquire new sperm structures. Functional interpretation of these new structure is sometimes possible. In the case of the parasitic Platyhelminthes, functional interpretation is not easy. Individuals of schistosomes (male and female) live in permanent pairs, and they have acquired small and simple spermatozoa. Individuals of *Diplozoon* also live in permanent pairs [138] (Fig. 5a), are fused, and the genital ducts communicate, and they have acquired a long and very complicated spermatozoon. Similar factors in *Schistosoma* and *Diplozoon* seem to have produced an evolution of the sperm structure in two completely opposite directions. The answer to this puzzling problem could be found if we consider that in both cases, the permanent pairing implies the absence of inter-individual sperm competition. The fact that the pair is made up of one male and one female in *Schistosoma* and of two hermaphrodites in *Diplozoon* does not seem to have an influence. Thus, in both cases, spermatozoa have not been subjected to selection in terms of performance such as speed or fertilization efficiency, and evolution could diverge in different directions.

The problem of an outgroup for the cladistic analysis of the parasitic Platyhelminthes

The problem of the recognition of a correct outgroup is central for the polarisation of characters. I will here concentrate on the interpretation of characters emerging from sperm studies, although it is clear that other structures may be highly useful. JUSTINE [98] has used, for defining an outgroup, the general system of EHLERS [50, 51]. The taxon Typhloplanida, which has spermatozoa with non-incorporated axonemes, was used as an outgroup for the Doliopharyngiophora, which comprise the Temnocephalidea, Dalyelliida, Udonellidea and Cercomeridea. It was noted [98] that the Dalyellioida have a very heterogeneous structure.

Source : MNHN, Paris

WATSON & ROHDE have systematically tried to find the outgroup for the Neodermata in the past year. *Pterastericola*, which has two incorporated axonemes was first proposed as an outgroup for the Neodermata [85]. Later, the analysis of spermiogenesis demonstrated that the fusion is not proximo-distal [242] and this hypothesis has been abandoned. DNA studies also concluded that Pterastericolidae are not the sister-group for the Neodermata [185].

Kronborgia (family Fecampiidae) was suggested as the outgroup [236, 237]. The spermatozoon of *Kronborgia* has two incorporated axonemes and a continuous row of cortical microtubules [237, 247, 248] and thus resembles that of the Monogenea Polyopisthocotylea. *Kronborgia* has a spermiogenesis with proximal centrioles, as in the parasitic Platyhelminthes. However, the proximo-distal fusion is not observed, there are no free flagella nor median cytoplasmic process, and the two axoneme grow within the elongating spermatid. The presence of a dense body makes its spermiogenesis close to most "Turbellaria". It is not certain that *Kronborgia* represents the taxon closest to the parasitic Platyhelminthes in term of ultrastructure of spermiogenesis. Should *Kronborgia* be considered the outgroup of the parasitic Platyhelminthes, this would require a re-examination of the polarity of certain characters, including the lateral microtubules (synapomorphy for the Polyopisthocotylea) which are present in *Kronborgia* and thus would become the plesiomorphic pattern. Recently, a study of 18s ribosomal RNA [186] has indicated that *Kronborgia* was not the sister-group of the Neodermata and could even be widely separate from them. However, this need confirmation from longer RNA sequences.

Udonella has a spermiogenesis with proximal centrioles, but there is no median cytoplasmic process nor fusion [189, 133, 189, 251]. This spermiogenesis is in fact similar to that of *Kronborgia*. However, the mature spermatozoon of *Udonella* has no microtubules [105]. Spermiogenesis in *Udonella* and *Kronborgia* resembles that found in certain Monogenea such as capsalids [114] or certain didymozoid digeneans [116] where a fusion is not observed because the axonemes grow within the elongating zone of differentiation. In these two latter cases, this characteristic should be interpreted as secondary reduction. The phyletic position of the Udonellidea is a matter of controversy: either outgroup for the Cercomeridea [30], or close to the Monopisthocotylea [183].

In a strictly spermatological point of view, we have, at present, no candidate for the role of outgroup of the parasitic Platyhelminthes. The proximo-distal fusion, with typical zone of differentiation, median cytoplasmic process and free flagella, is found only in the Aspidogastrea, Digenea, Monogenea Polyopisthocotylea, Gyrocotylidea, Amphilinidea, and certain Eucestoda. The analogies of the zone of differentiation in the Monogenea Monopisthocotylea [97, 98] and in certain Eucestoda [21] with the basic structure of the parasitic Platyhelminthes suggest that, although they lack a true proximo-distal fusion, these taxa may be considered closely related. However, the proximo-distal fusion has not been found in any taxon more primitive than the Cercomeridea.

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Source : MNHN, Paris

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Source : MNHN, Paris

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Source : MNHN, Paris

Spermiogenesis, Spermatozoa and Phyletic Affinities in the Cestoda

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ABSTRACT

Comparative ultrastructural studies of spermiogenesis and/or spermatozoa of 43 species of cestodes lead us to conclude that flagellar rotation and the proximodistal fusion of one or two flagella with the median cytoplasmic extension is a plesiomorphic character in the Eucestoda and that the absence of flagellar rotation is a synapomorphy for the Cyclophyllidea. We consider the presence of the crested-like body at the anterior extremity of the spermatozoa of cestodes as a synapomorphy for the Eucestoda. We also confirm the absence of mitochondria as a synapomorphy for the Eucestoda. Previous phylogenetic diagrams are critically reviewed.

RÉSUMÉ

Spermiogenèse, spermatozoïdes et affinités phylétiques chez les Cestoda.

L'étude ultrastructurale comparée de la spermiogenèse et/ou du spermatozoïde de 43 espèces de cestodes nous a permis de considérer la rotation flagellaire et la fusion proximo-distale du ou des flagelles spermatiques avec une expansion cytoplasmique médiane comme un caractère plésiomorphe des Eucestodes et l'absence de rotation flagellaire comme une synapomorphie des Cyclophyllidea. Nous considérons la présence de corps en crête à l'avant du spermatozoïde comme une synapomorphie des Eucestodes. Nous confirmons également l'absence de mitochondrie comme une synapomorphie des Eucestodes. Les schémas phylétiques précédents sont révisés de manière critique.

Cestodes have been described in all vertebrates except agnathans [44]. They now comprise nearly 4 000 species spread over 600 genera, 63 families and 13 orders. The different authors interested in their phylogenesis on the basis of their morphological characters [14, 16, 19, 20, 22], frequently came to contradictory conclusions. The absence of mitochondria in spermatozoa has been considered as a synapomorphy of the Cestodes [17, 18], and the spiral coil of the cortical spermatic microtubules as a synapomorphy of the Cyclophyllidea [26]. In the present work, we propose two new synapomorphies, one for the Eucestoda and one for the Cyclophyllidea. Moreover, we critically debate the two last phylogenetic diagrams proposed by EUZET *et al.* [20] and BROOKS *et al.* [16].

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Source : MNHN, Paris

MATERIAL AND METHODS

We have studied the ultrastructure of spermiogenesis and/or the spermatozoa of 14 species of cestodes (Table 1). The specimens of the different species were gathered from the intestines of their respective hosts (birds or mammals), naturally infested, then placed, alive and active, in a physiological saline solution (9 ‰ NaCl). Portions of strobile, 3 to 6 cm long, consisting of mature proglottids, were quickly taken, then stretched out with a brush soaked in cold (4°C) 2.5 % glutaraldehyde in a 0.1 M sodium cacodylate buffer at pH 7.2. The genital apparatus was removed under a binocular microscope, fixed for about 24 hours in glutaraldehyde at 4°C, rinsed for one night in the sodium cacodylate buffer, post-fixed for one hour with cold 1% osmium tetroxide, dehydrated with ethanol and propylene oxide, then embedded in epon. Ultrathin sections were cut on a Porter-Blum MT1 and Reichert-Jung Ultracut-E ultramicrotomes, then stained with uranyl acetate and lead citrate. They were examined in Siemens Elmikop 101 and JEOL 100 CX II electron microscopes.

RESULTS

The figures 1 to 5 and Table 1 present our observations and those of other authors on spermiogenesis and/or the spermatozoa of cestodes. Whatever the cestode may be, spermiogenesis always begins by the formation of a differentiation zone (Figs 1a, 2a, 3a, 4a). This is delimited at the proximal extremity by arched membranes, and bordered by cortical microtubules. This contains one or two centrioles separated (Figs. 1a, 2a) or not (Figs 3a, 4a) by an intercentriolar body and surmounted (Figs. 1a, 2a) or not (Figs 3a, 4a) by striated roots or a centriolar-adjunct (Fig. 4a). Each centriole very rapidly gives rise to a flagellum that grows within (Figs 4b-c) or external to (Figs 1b, 2b, 3b) the differentiation zone. Subsequently, the flagellum (or the flagella) undergoes (Figs 1c, 2c) or not (Figs 3c, 4c) a rotation, becomes parallel to the cytoplasmic extension, and fuses with it. After the migration of the nucleus into the differentiation zone and the formation of one or many crested-like bodies, the ring of arched membranes narrows until the spermatid detaches itself from the residual cytoplasm. The mature spermatozoon of the cestodes lacks mitochondria, is filiform and is tapered at both extremities (Fig. 5). Its anterior extremity exhibits an apical cone of electron dense material and one or many crested-like bodies. The cytoplasm contains proteinaceous material and a nucleus coiled or not in a spiral around the axoneme. The proteinaceous material may be arranged in four different forms: granulations, rods making intracytoplasmic walls, a periaxonemal sheath, submicrotubular thickenings (Fig. 5).

DISCUSSION

During spermiogenesis of the Cercomeridea (Aspidobothrea, Digenea, Monogenea, Gyrocotylidea, Amphilinidea and Eucestoda), the flagellum (or the flagella) of old spermatids undergoes a rotation and becomes parallel to a median cytoplamic extension with which it fuses. This proximodistal fusion as it is termed [26, 27] is not found in the Turbellaria [26, 56]. In the cestodes in particular, it is encountered in the Tetraphyllidea-Onchobothriidae [33, 37], the Tetraphyllidea-Phyllobothriidae [36], the Tetrarhynchidea [51], the Proteocephalidea [50, 51], the Pseudophyllidea [54] and the Caryophyllidea [51]. The proximodistal fusion has recently been proposed as a synapomorphy for all the Cercomeridea [26]. Nevertheless, in three Cyclophyllidea, *Thysaniezia ovilla* [13], *Hymenolepis nana* [3] and *Aporina delafondi* [8], the single flagellum grows directly into the spermatid body. In three other Cyclophyllidea, *Nematotaenia chantalae* [38], *Mathevotaenia herpestis* [7] and *Raillietina (Raillietina) tunetensis* [9], the flagellum grows outside the differentiation zone, parallel to the cytoplasmic extension and then fuses with it before nuclear migration. Dealing with the existence or not of a flagellar rotation during spermiogenesis, we can distinguish in the Cercomeridea two types of spermiogenesis. The former which involves a flagellar rotation and a proximodistal fusion, has been supposed to exist in all the Cercomeridea [26]. The latter which is characterized by an absence of flagellar rotation, has been reported in the six Cyclophyllidea which we have previously cited [3, 7, 8, 9, 13, 38]. In the present work, we consider the absence of flagellar rotation as an apomorphic character for all the Cyclophyllidea.

Source : MNHN, Paris

TABLE 1. — Cestodes whose spermatozoa have been studied by electron microscopy. The references quoted contain data on spermiogenesis (*), the presence of one (+) or more than one (++) crested-like bodies and the presence of two axonemes (2a).

Taxon	Reference
Haplobothrioidea	
Haplobothriidae	
Haplobothrium globuliforme (2a)*	[32]
Pseudophyllidea	
Bothriocephalidae	
Bothriocephalus clavibothrium (2a)*	[54]
Cyathocephalidae	
Bothrimonus sturionis	[31]
Caryophyllaeidae	
Monobothrium wageneri	[34]
Glaridacris catostomi	[53]
Diphyllobothriidae	
Diphyllobothrium latum (2a)	[15]
Duthiersia fimbriata + (2a)	[25]
Proteocephalidea	
Proteocephalidae	
Proteocephalus longicollis (2a)	[52]
Sandonella sandoni +	[10]
Tetrarhynchidea	
Lacistorhynchidae	
Lacistorhynchus tenuis (2a)	[48]
Diphyllidea	
Echinobothriidae	
Echinobothrium typus +*	[1]
Echinobothrium brachysoma +*	[1]
Echinobothrium affine (2a)*	[1]
Echinobothrium harfordi +*	[1]
Tetraphyllidea	
Phyllobothriidae	
Pseudanthobothrium hanseni +	[31]
Phyllobothrium gracile +*	[36]
Echeneibothrium beauchampi +	[40]
Onchobothriidae	
Acanthobothrium filicolle filicolle + (2a)*	[35, 37]
Acanthobotrium filicolle benedenii + (2a)	[39]
Onchobothrium uncinatum + (2a)	[39]
Trilocularia acanthiaevulgaris + (2a)*	[33]
Cyclophyllidea	
Anoplocephalidae	
Inermicapsifer madagascariensis++	[11, 12, 49]
I. guineensis ++	[11, 12]
Moniezia expansa ++	[4, 46]
M. benedeni ++	[4]
Monoecocestus americanus	[31]
Stilesia globipunctata +	[2]
Avitellina centripunctata +	[6]
Thysaniezia ovilla +*	[13]
Mathevotaenia herpestis +*	[7]
Aporina delafondi +*	[8]
Oochoristica agamae	[55]
Catenotaeniidae	
Catenotaenia pusilla	[47]
Hymenolepididae	
Hymenolepis diminuta *	[2, 6, 30, 42, 43, 45]
Hymenolepis nana ++*	[3, 43]
H. microstoma	[47]
Retinometra serrata ++	[5]
Taeniidae	
Echinococcus granulosus	[41]
Taenia hydatigena *	[21]
Davaineidae	
Raillietina (Raillietina) tunetensis ++*	[9]
Cotugnia polyacantha ++	[11]
Nematotaeniidae	
Nematotaenia chantalae +*	[38]
Cylindrotaenia hickmani	[23, 24]

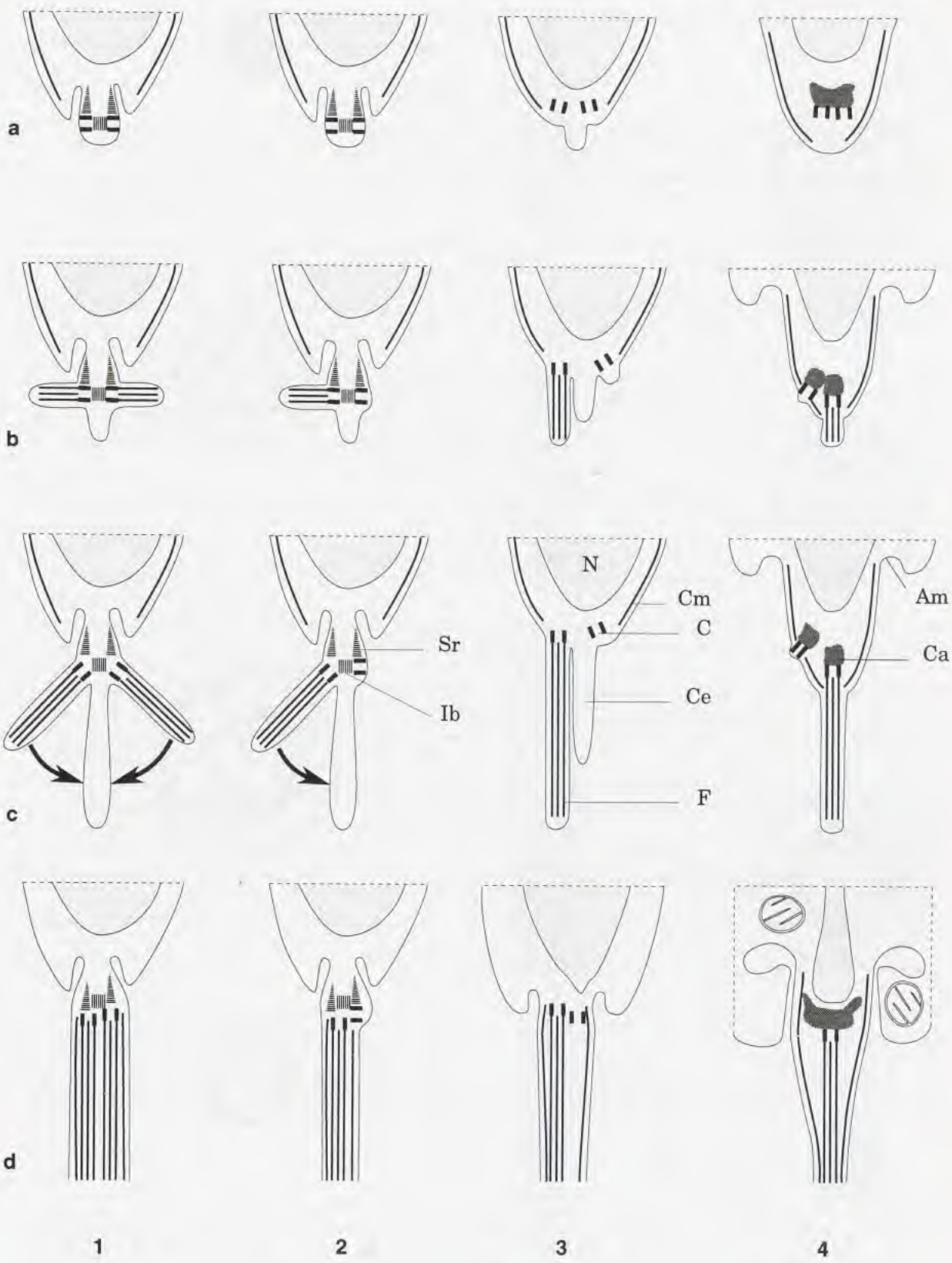
Source : MNHN, Paris

One or many crested-like bodies have been described at the anterior extremity of the spermatozoa of 26 species of cestodes: one Pseudophyllidean, one Proteocephalidean, three Diphyllidea, seven Tetraphyllidea and 14 Cyclophyllidea (Table 1). Although their presence has not been reported by some authors [24, 29, 42, 43], we have observed them in their illustrations. In a Caryophyllidean, *Glaridacris catostomi* [53], a Haplobothrioidean, *Haplobothrium globuliforme* [32] and a Tetrarhynchidean, *Lacistorhynchus tenuis* [48], these formations have neither been described nor figured. In fact, it is not easy to detect them owing to their very small size. Thus, in spite of these few "exceptions", and although our knowledge is still limited to a small number of species, we believe that the crested-like body should be considered as a synapomorphy for all cestodes. Moreover, we consider the presence of a crested-like body in the spermatozoa of one species of monopistocotylean Monogenea, *Calceostoma* sp. [28], as a simple phenomenon of convergence.

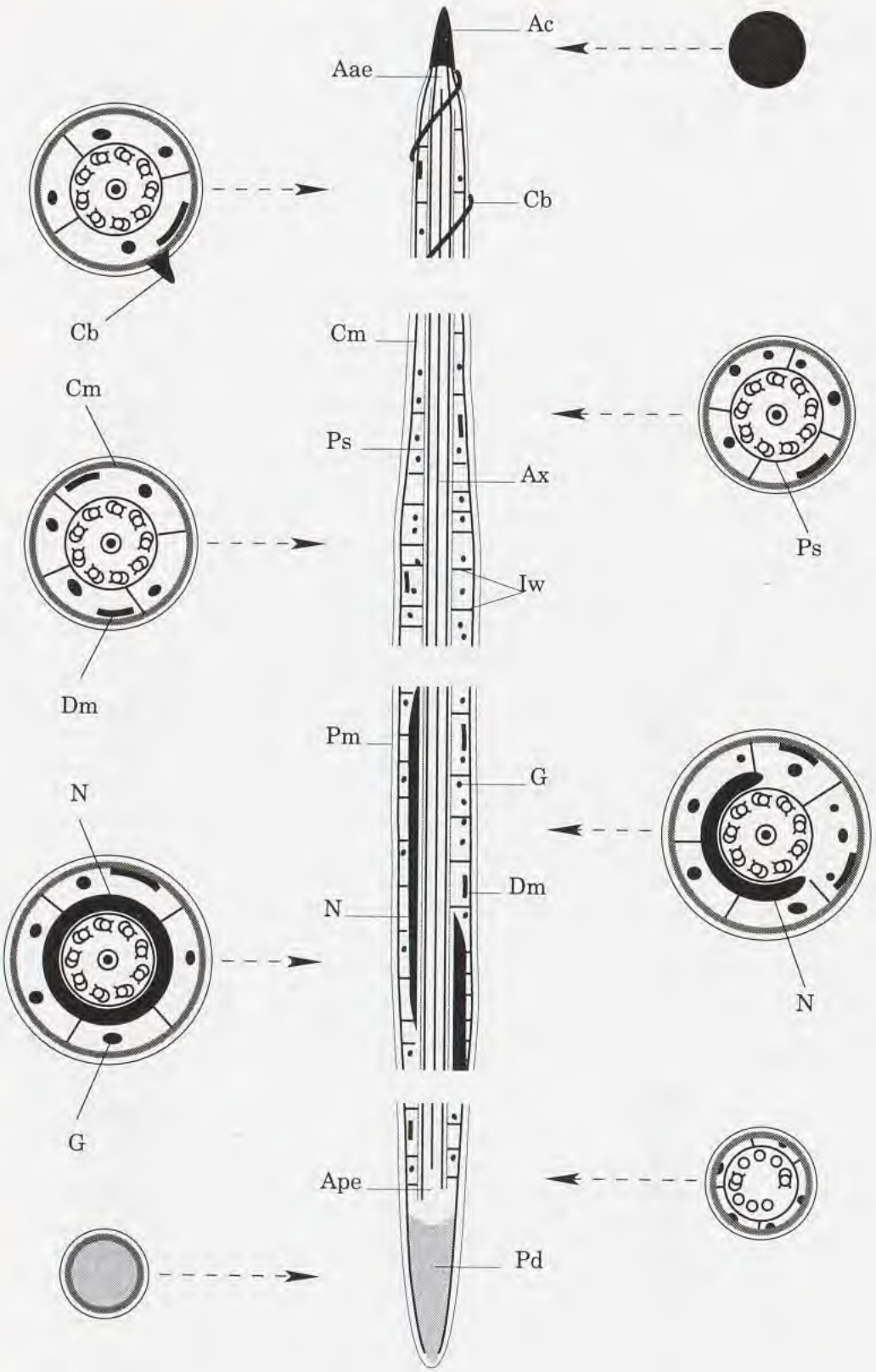
The phylogenetic systematics of the cestodes are still poorly understood and are much debated. EUZET *et al.* [20] thought that the presence of a single axoneme in the cestode spermatozoon is an evolved character. Thus, they considered the Cyclophyllidea as derived from the Proteocephalidea, then the Proteocephalidea and the Tetraphyllidea-Phyllobothriidae as issued from the Tetraphyllidea-Onchobothriidae and lastly the Pseudophyllidea and the Tetrarhynchidea as coming from the Haplobothrioidea. The Caryophyllidea and the Diphyllidea were of unknown origin. On the other hand, FREEMAN [22] estimated the Tetraphyllidea-Phyllobothriidae as the ancestors of the Cyclophyllidea, taking into consideration their post-embryonic development. Schmidt [44], considered the Haplobothrioidea and the Tetrarhynchidea respectively as families belonging to the Pseudophyllidea and the Trypanorhynchidea. BROOKS *et al.* [16], using 12 synapomorphic characters, subdivided the cestodes into five orders: Pseudophyllidea, Nippotaeniidea, Proteocephalidea, Lecanicephalidea and Tetraphyllidea. Additionally, they included the Caryophyllidea, the Cyclophyllidea and the Trypanorhynchidea, respectively, in the Pseudophyllidea, the Proteocephalidea and the Tetraphyllidea. The phylogenetic systematic diagram of BROOKS *et al.* (1991) [16] involves the coexistence of spermatozoa bearing one or two axonemes in the same order (Table 1), thus showing a contradiction between spermatological and morphological characters. Moreover, their propositions do not clarify the evolution of the number of axonemes within the Cestodes. When considering the pattern of the posterior extremity of the spermatic flagella, some important differences between the Proteocephalidea and the Cyclophyllidea can be pointed out. In the Cyclophyllidea *Thysaniezia ovilla* [13], *Stilesia globipunctata* [2], *Hymenolepis nana* [3], *Moniezia expansa* and *Moniezia benedeni* [4], *Retinometra serrata* [5], *Aporina delafondi* [8] and *Raillietina (R.) tunetensis* [9], the central element of the axoneme disappears before the simplification of doublets into singlets. On the other hand, in the Proteocephalidean *Sandonella sandoni* [10], the posterior extremity of the spermatozoon consists of the axonemal central element, surrounded by nine singlets that correspond to the A microtubules which are in close contact with the plasmic membrane.

FIGS 1-4. — Attempted reconstruction of the different types of spermiogenesis in the Cestodes. Am, arched membranes; C, centriole; Ca, centriolar-adjunct; Ce, cytoplasmic extension; Cm, cortical microtubules; F, flagellum; Ib, intercentriolar body; Sr, striated root. **1 a-d:** First type of spermiogenesis with flagellar rotations (c) and proximodistal fusions (d) as described in the Tetraphyllidea-Onchobothriidae [30, 33], the Proteocephalidea [48, 49] the Tetrarhynchidea [49] and the Pseudophyllidea [52]. **2 a-d:** Second type of spermiogenesis with a flagellar rotation (c) and a proximodistal fusion (d) as described in the Tetraphyllidea-Phyllobothriidae [32] and the Caryophyllidea [49]. **3 a-d:** Third type of spermiogenesis without flagellar rotation but with a proximodistal fusion (d). The single flagellum grows outside but parallel to the cytoplasmic extension (c) then fuses with it (d) as described in some species of the Cyclophyllidea: *Nematotaenia chantalae* [34], *Mathevotaenia herpestis* [7] and *Raillietina (R.) tunetensis* [9]. **4 a-d:** Fourth type of spermiogenesis with neither flagellar rotation nor proximodistal fusion. The single flagellum grows inside the cytoplasmic extension (b-d) as described in some other species of the Cyclophyllidea: *Thysaniezia ovilla* [13], *Hymenolepis nana* [3] and *Aporina delafondi* [8].

Source : MNHN, Paris



Source : MNHN, Paris



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Source : MNHN, Paris

Thus, it becomes obvious that the phylogenetic systematics of the Cestodes is a complex subject and requires serious revision.

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FIG. 5. — Longitudinal and cross sections of the different constitutive elements of the spermatozoa of the cestodes. Aae, axonemal anterior extremity; Ac, apical cone; Ape, axonemal posterior extremity; Ax, axoneme; Cb, crested-like body; Cm, cortical microtubules; Dm, submicrotubular electron dense material; G, proteinaceous granules; Iw, intracytoplasmic walls; N, nucleus; Pd, posterior electron dense material; Pm, plasma membrane; Ps, periaxonemal sheath.

Source : MNHN, Paris

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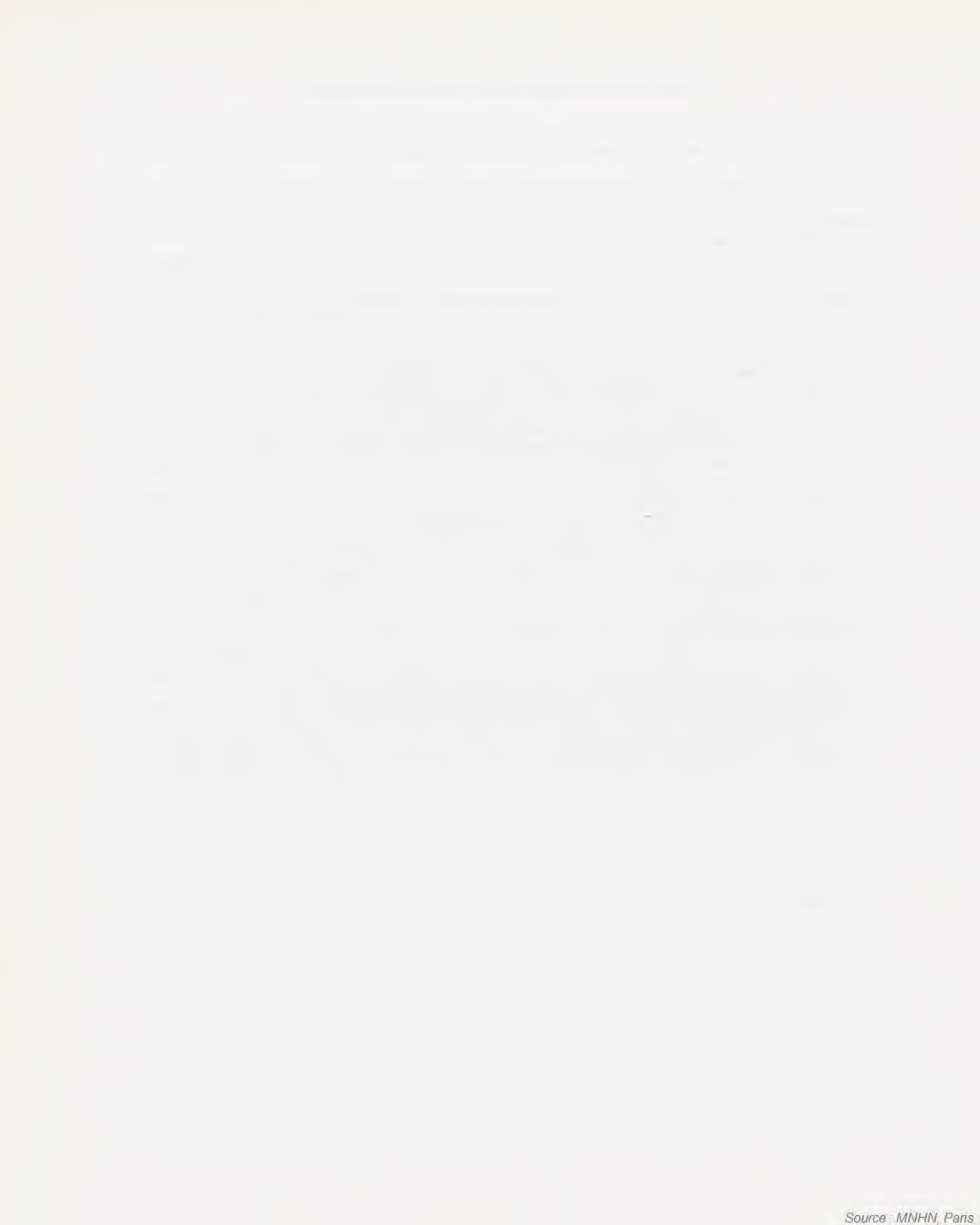
Source : MNHN, Paris



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Immunocytochemistry of Tubulin in Spermatozoa of Platyhelminthes

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ABSTRACT

Indirect immunofluorescence and electron microscope post-embedding immunocytochemistry were used to detect tubulin in spermatids and spermatozoa of various Platyhelminthes. General sperm morphology is exemplified by conventional electron microscope observations on *Gorgodera* sp. and *Haematoloechus* sp. The utility of tubulin indirect immunofluorescence for the understanding of the filiform spermatozoa of Platyhelminthes is underlined by a comparison between *Echinostoma* (Digenea) and *Symsagittifera* (Acoela). This technique might be useful for further comparative research with phylogenetic implications. In the digenean *Echinostoma* sp., alpha- and beta-tubulin are detected in the doublets of the 9+"1" axonemes and in the cortical singlet microtubules, but alpha-acetylated-tubulin is present in the axonemal doublets and absent in the cortical microtubules. The central core of the Platyhelminthes 9+"1" axoneme is not labelled by any of the three monoclonal anti-tubulin antibodies used (anti-alpha-, anti-beta- and anti-alpha-acetylated-tubulin).

RÉSUMÉ

Immunocytochimie de la tubuline dans les spermatozoïdes de Plathelminthes

L'immunofluorescence indirecte et l'immunocytochimie ultrastructurale post-inclusion ont été utilisées pour détecter la tubuline dans les spermatides et les spermatozoïdes de Plathelminthes variés. La morphologie générale des spermatozoïdes est explicitée par des observations de microscopie électronique conventionnelle de *Gorgodera* sp. et *Haematoloechus* sp. L'intérêt de l'immunofluorescence indirecte de la tubuline pour la compréhension de la structure des spermatozoïdes filiformes des Plathelminthes est souligné par une comparaison entre *Echinostoma* (Digenea) et *Symsagittifera* (Acoela). Cette technique pourrait être utile pour une recherche comparée à but phylogénique. Chez *Echinostoma* sp., l'alpha-tubuline et la beta-tubuline sont détectées dans les doublets des axonèmes 9+"1" et dans les singulets corticaux, mais la tubuline acétylée est présente seulement dans les doublets des axonèmes et absente dans les singulets corticaux. L'élément central des axonèmes 9+"1" de Plathelminthes n'est marqué par aucun des trois anticorps utilisés (contre la tubuline alpha, beta, et alpha-acétylée).

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Source : MNHN, Paris

Tubulin, the protein constituting the microtubules, is one of the major proteins in spermatozoa which have axonemes and/or microtubules. Tubulin shows a high degree of heterogeneity owing to two factors, genetic diversity [21] and posttranslational modifications: acetylation [19], glutamylation [8], detyrosylation [33], phosphorylation [10, 22] and the recently discovered polyglycylation [28]. Because tubulins are well conserved during evolution [1] and posttranslational modifications widely distributed among various species, antibodies raised against tubulin or posttranslational modifications of one species generally recognize the same epitope in other species and can thus be used as tool to investigate tubulin properties in many systems.

In this study, we demonstrate that different anti-tubulin antibodies can differentiate distinct subcellular population of microtubules in spermatozoa of Platyhelminthes. In addition, immunocytochemistry of tubulin gives valuable information about the general morphology of the spermatozoon, and new insight into the composition of the 9+"1" axoneme of the Platyhelminthes. These results might be of phylogenetic interest when further comparative data are obtained.

MATERIAL AND METHODS

Material. Digeneans were collected from hosts which can be kept easily in the laboratory. *Gorgodera* sp. and *Haematoloechus* sp. were collected from commercial frogs (*Rana* sp.) originating from various localities of Eastern Europe. *Echinostoma liei* and *Echinostoma caproni* were collected from experimentally infested laboratory mice. The Acoela *Symsagittifera schultzei* was extracted from sand collected in Roscoff, France.

Light microscope immunocytochemistry. Germ cells were obtained by squashing worms in a drop of PBS (phosphate buffer saline, Sigma) on a pit slide previously washed with alcohol and acetone. Slides were allowed to dry for 1 h under a fan, then kept at 4°C and processed within 24 h. Cells were permeabilized in acetone (10 min) and rinsed (PBS, 3 x 5 min). Non-specific antigens were blocked with 2% Bovine Serum Albumin (Sigma) in PBS (BSA-PBS) for 45-90 min at room temperature. A monoclonal anti-tubulin antibody (anti-alpha-tubulin, clone DM 1A, Sigma, or anti-beta-tubulin, clone TUB 2.1, Sigma or anti-acetylated-tubulin, clone 6-11B-1, Sigma [26]) diluted at 1/200 to 1/600 in BSA-PBS was applied for 40 min at room temperature. After rinsing (PBS 3 x 5 min), a FITC-conjugated antibody (Goat anti-mouse, Nordic, 1/40 in PBS) was applied for 40 min at room temperature. For double labelling, supplementary steps were added as follows: anti-tubulin polyclonal antibody (Sigma, ref. T-3526) diluted at 1/40 in BSA-PBS applied for 40 min at room temperature, rinsed (PBS 3 x 5 min) and followed by a TRITC-conjugated antibody (Goat anti-rabbit, Nordic, 1/40 in PBS) for 40 min at room temperature. The nuclear dye Hoechst 33258 (1 µg/ml in PBS, 10 min) or DAPI (1 µg/ml, 10 min) was sometimes used for labelling the nucleus. After a final rinse (PBS 3 x 5 min), mounting was done in Citifluor (Citifluor Ltd, London, UK) and slides were sealed with nail enamel. Controls were done by omitting the first antibody or by using a non-relevant mouse antibody. Observations were made with a Nikon Optiphot epifluorescence microscope equipped with a mercury lamp and a single band Nikon filter for FITC channel (B-2A), TRITC channel (G-2A) or Hoechst channel (UV-1A), or a double-band (FITC/TRITC) Omega filter (XF 52).

Conventional electron microscopy. Living specimens cut into pieces were fixed for 1 h in 2% glutaraldehyde in a buffer solution of 0.1 M sodium cacodylate at pH 7.2 at 4°C. After rinsing in the same buffer, the worms were postfixed for 1 h in 1% osmium tetroxide in the same buffer, dehydrated in ethanol and propylene oxide, and embedded in SPURR's medium or in Epon. Ultrathin sections were contrasted with DADDOW's method [7] or with conventional lead citrate and uranyl acetate, and observed with a Hitachi H600 electron microscope.

Post-embedding electron microscope immunocytochemistry. Living worms were fixed for 1 h in 2% glutaraldehyde in a buffer solution of 0.1 M sodium cacodylate at pH 7.2 at 4°C. After rinsing in the same buffer (3 x 10 min), the worms were embedded in LR White resin (medium grade). The resin was polymerized in tightly capped gelatin capsules for 10 h at 60°C. Ultrathin sections were placed on nickel or gold grids. Grids were rinsed (PBS, 3 x 5 min), and non-specific antigens were blocked with goat immunoglobulins (Sigma) diluted at 1/30 in PBS for 1h. A monoclonal anti-tubulin antibody (anti-alpha-tubulin, clone DM 1A, Sigma, or anti-beta-tubulin, clone TUB 2.1, Sigma or anti-acetylated-tubulin, clone 6-11B-1, Sigma [26]) diluted at 1/100 in PBS, was applied for 40 min at room temperature. After rinsing (PBS, 3 x 5 min), a gold-conjugated antibody (Goat anti-mouse, 15 nm gold beads, 1/20 in PBS) was applied for 1 h at room temperature. After a final rinse (PBS 3 x 5 min, then distilled water, 3 x 5 min), the grids were dried on Whatman paper, stained with DADDOW's method [7] and observed with a Hitachi H600 electron microscope.

Source : MNHN, Paris

RESULTS

Conventional electron microscopy (Fig. 1)

Results of conventional electron microscopy are briefly presented in this paper to show the structure described below with electron microscope immunocytochemistry. In the digeneans, spermiogenesis is homogeneous in most species [16, 17]. The spermatid shows a protrusion called the zone of differentiation (Fig. 1a). At the distal extremity of this zone of differentiation, three processes elongate: a median cytoplasmic process, and two flagella. The median cytoplasmic process contains singlet microtubules aligned below the membrane (Fig. 1b). Each flagellum contains an axoneme with the 9+"1" trepaxonematan structure (Fig. 1c). The spermatozoon (Fig. 1c) is later produced by the fusion of the three processes, and contains two 9+"1" axonemes, dorsal and ventral microtubules, an elongate mitochondrion and an elongate nucleus. The 9+"1" axonemes (Fig. 1b, c) do not contain the pair of central microtubules of the almost ubiquitous 9+2 axonemes but have, instead, a solid central core.

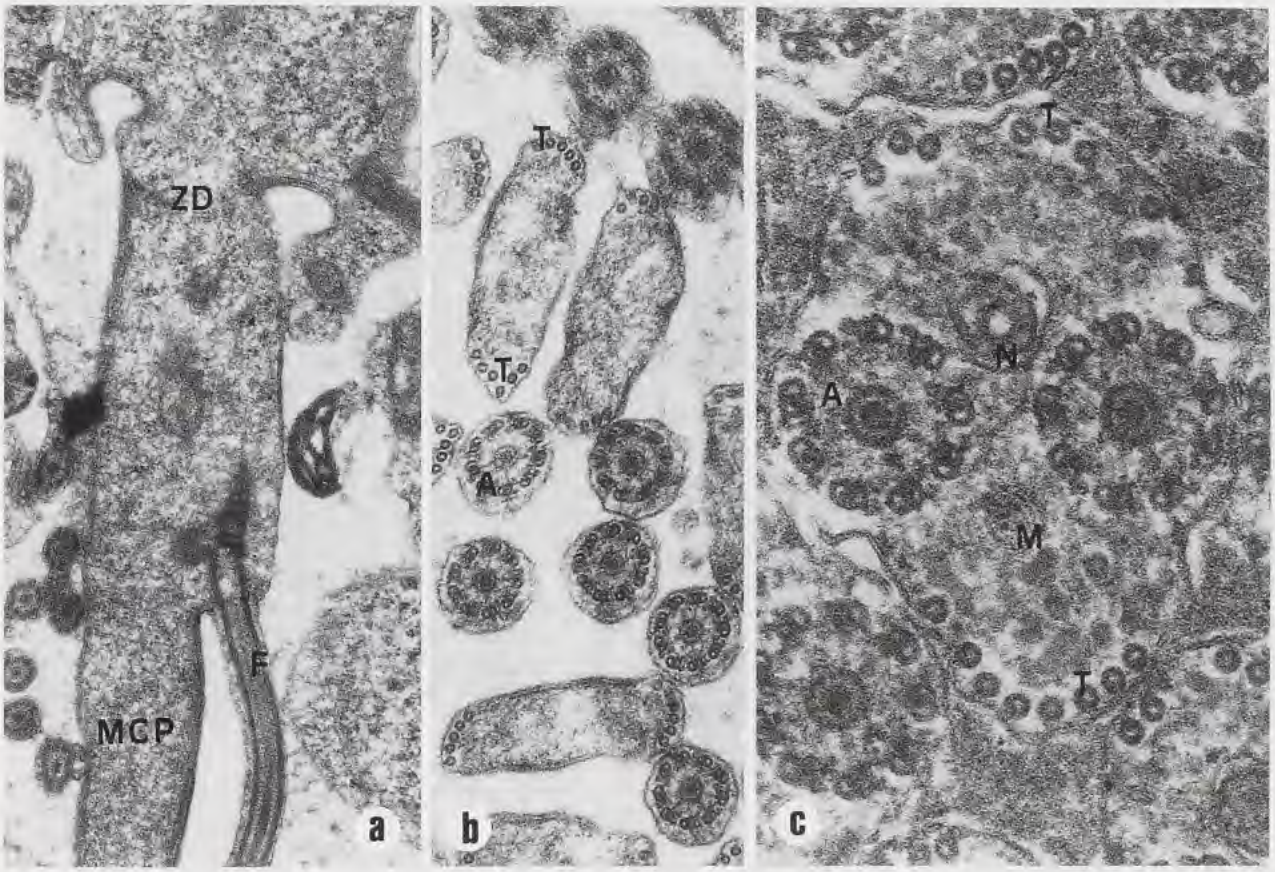

FIG. 1. — Conventional electron microscopy of digenean spermatozoa. **a, b:** *Gorgodera* sp. **a:** Zone of differentiation (ZD) in spermatid, longitudinal section showing median cytoplasmic process (MCP) and flagella (one sectioned here, F) which will fuse to produce the mature spermatozoon. **b:** Median cytoplasmic process with singlet microtubules (T) and free flagella containing 9+"1" axonemes (A), transverse section. **c:** *Haematoloechus* sp., mature spermatozoon, transverse section. A, axonemes with 9+"1" trepaxonematan pattern; M, mitochondrion; N, nucleus; T, dorsal and ventral microtubules. a, x 20 000; b, x 48 000; c, 125 000.

Source : MNHN, Paris

Light microscope immunocytochemistry (Fig. 2)

Digenean spermatozoa, in all species studied, are long, filiform cells. Spermatozoa contain two parallel axonemes. These are labelled by the anti-tubulin antibodies (Fig. 2a) and appear as two closely parallel lines in some cases, but often as a single thick line. In some cases, the process of drying and re-hydrating during processing partly alters the cell and, in certain regions of the spermatozoon, two lines, sometimes widely separate, are visible. The use of various antibodies and the technique of double labelling (Fig. 2b, c) reveal that tubulin epitopes are different in these two microtubular structures. The two lines are labelled by the polyclonal anti-tubulin (Fig. 2b), the anti-alpha-tubulin (Fig. 2a) and the anti-beta-tubulin monoclonal antibodies, but the monoclonal anti-acetylated-alpha-tubulin antibody labels only one line (Fig. 2c). This shows that two distinct subpopulations of microtubules are present, but interpretation of the nature of these two lines requires the use of electron microscope immunocytochemistry, described below.

Acoel spermatozoa also contain two parallel axonemes, which do not show the 9+"1" pattern but instead a 9+0 or 9+2 pattern [27]. Immunocytochemistry (Fig. 2d) reveals that these axonemes are widely separated and show longitudinal undulations. The axonemes are visible with the three antibodies used.

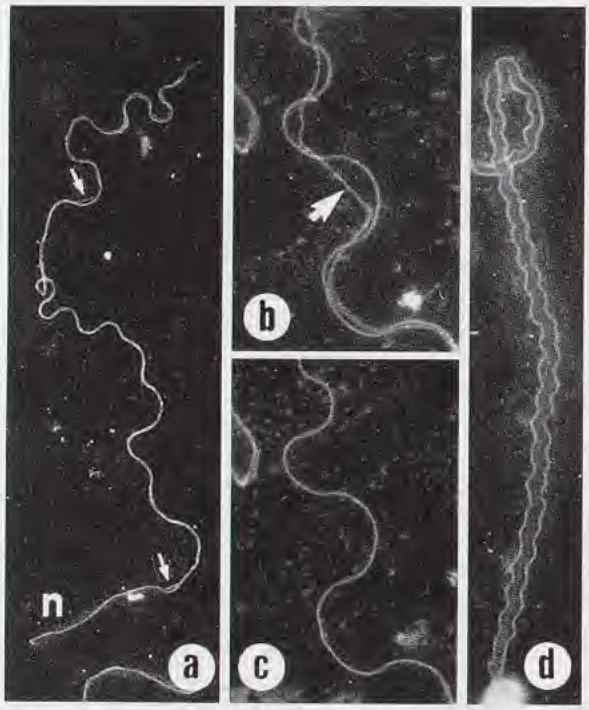

FIG. 2. — Light microscope immunocytochemistry of platyhelminth spermatozoa. **a-c:** *Echinostoma caproni* (Digenea). **a:** View of spermatozoon showing filiform pattern. Anti-alpha-tubulin labelling. n, nucleus (evidenced by a nuclear dye, not shown here). Two lines are visible in limited regions of the cell (arrows). **b, c:** double labelling with polyclonal anti-tubulin antibody (b) and monoclonal anti-acetylated-alpha-tubulin antibody (c) in a region where two lines are visible. Acetylated-alpha-tubulin is detected in one line only. Interpretation of these labelling requires electron microscope immunocytochemistry (see Fig. 3 and Discussion). **d:** *Symsagittifera schultzei* (Acoela), anti-alpha-tubulin antibody. The two axonemes are widely separated and undulate. a, d, x 600; b, c, x 1 200.

Electron microscope immunocytochemistry. (Fig. 3)

Axonemal microtubules and cortical singlet microtubules show striking differences in their labelling. The anti-alpha tubulin and the anti-beta-tubulin antibodies label the cortical singlets together with the axonemal microtubules (Fig. 3a, b, d, e). The anti-acetylated-alpha-tubulin antibody (Fig. 3c, f, g) labels only the axonemal microtubules ; the cortical singlets are not labelled.

None of the antibodies labels the central core of the 9+"1" axoneme (Fig. 3a-g).

Source : MNHN, Paris

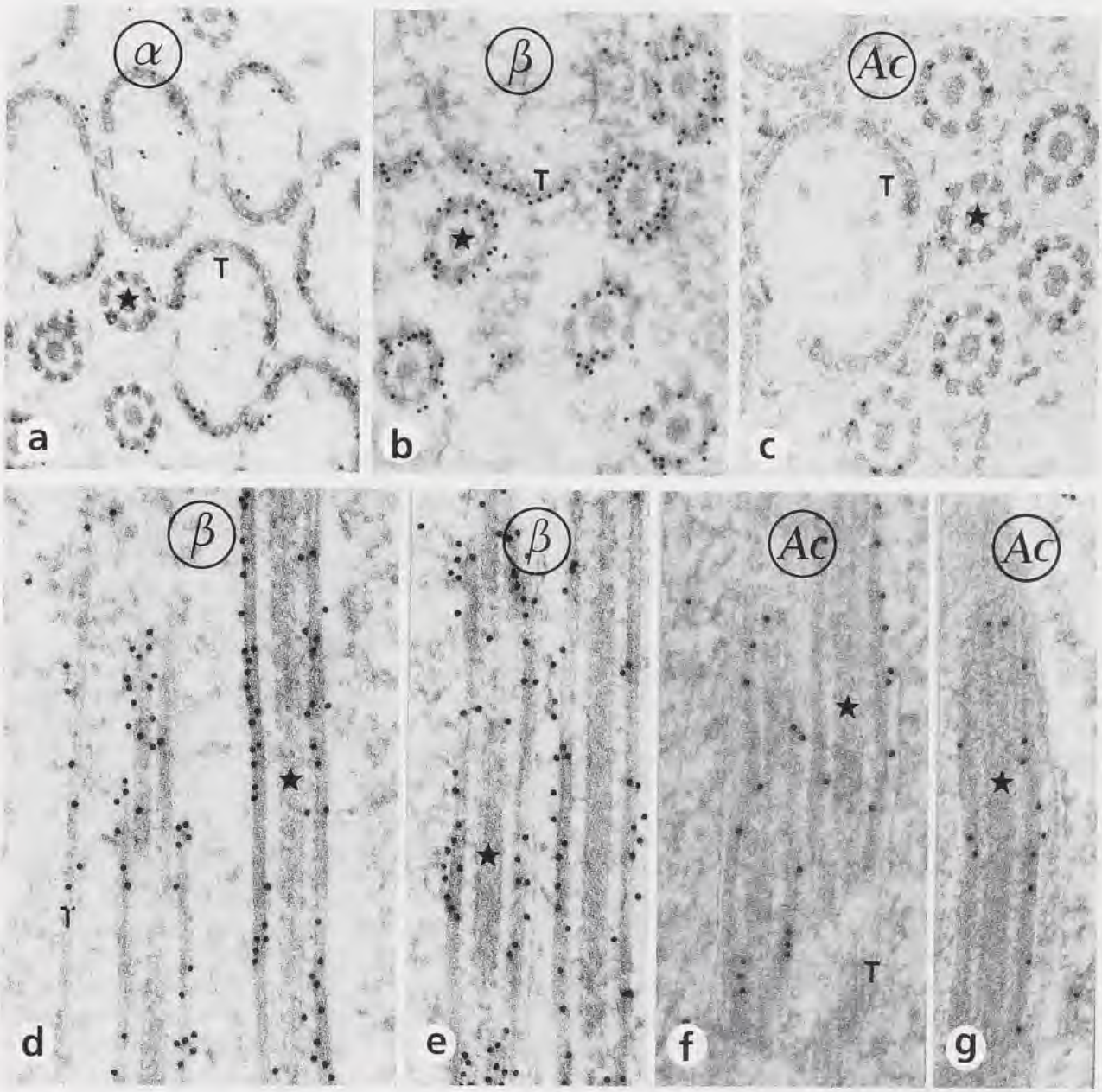

FIG. 3. — Electron microscope immunocytochemistry of spermatids of *Echinostoma liei* (Digenea). **a-c:** Median cytoplasmic process and flagella, transverse section (compare with figure 1b). **a:** anti-alpha-tubulin. **b:** anti-beta-tubulin. **c:** anti-acetylated-alpha-tubulin. **d-g:** Spermatids, longitudinal section. **d, e:** anti-beta-tubulin; **f, g:** anti-acetylated-alpha-tubulin. Axonemal microtubules are labelled in each case, but singlet microtubules (T) in the median cytoplasmic process are labelled by the anti-alpha and anti-beta-tubulin antibodies but not by the anti-acetylated-alpha-tubulin antibody. The central core (star) of the 9+"1" axoneme is never labelled by the anti-tubulin antibodies. a-c, x 50 000; d-f, 70 000; g, x 60 000.

Source : MNHN, Paris

DISCUSSION

Tubulin immunocytochemistry: an innovative technique for understanding the general morphology of spermatozoa

Spermatozoa of Platyhelminthes are generally very long and filiform [16, 17], without a "head" and a "tail", and thus are chiefly described by means of cross sections. Ordering the various cross sections along a scheme of the sperm body from random sections is not trivial and serial sectioning is very time consuming. Anti-tubulin immunocytochemistry, although a routine technique in cell biology, has not previously been used for purely morphological studies. This technique allows a view of the whole filiform spermatozoon and of the arrangement of the microtubular organelles along its length. The two examples presented in Figure 2 show the differences between a "turbellarian" spermatozoon, here an acoelan, with almost independent axonemes which undulate along the cell, and a digenean, with very close axonemes. Anti-tubulin immunocytochemistry is an innovative technique for the understanding of spermatozoa in two very different cases: filiform spermatozoa which are almost "two-dimensional" such as those of Platyhelminthes (this study), and complex three-dimensional spermatozoa such as those of crustaceans [24, 25, 34].

Tubulin subpopulations in Platyhelminthes spermatozoa: axonemes versus cortical microtubules

The results of electron microscope immunocytochemistry allows the interpretation of the double labelling observed in *Echinostoma* with indirect immunofluorescence (Fig. 2b, c) as follows: one line, labelled by all the antibodies used, is made up of the two closely associated axonemes; the other line, labelled by all antibodies except the anti-acetylated-alpha-antibody, represents the singlet microtubules.

The location of tubulin in Platyhelminthes has received little attention [32]. Results presented here show that different tubulin epitopes are located in the axonemes and in the cortical microtubules. The detection of alpha and beta-tubulin in the axonemes was expected, since alpha and beta tubulin are constituents of the microtubules. It, however, reveals that anti-tubulin antibodies developed against non-Platyhelminthes antigens can be used for this purpose.

The differential location of acetylated tubulin (present in axonemes, absent in cortical microtubules) is a more interesting finding. Posttranslational varieties of tubulin have been investigated in spermatozoa of only a few species: glutamylated in mammals [11, 18], tyrosinated in sea urchin, man [13] and rat [15], polyglycylated in *Drosophila* [2]. Acetylated tubulin has been detected in male germ cells of sea urchin [26], insects [26, 35], fish [20], and various mammals including man [11, 12, 15], but is apparently absent in the transient microtubules of aflagellate nematode spermatids (MANSIR & JUSTINE, this volume) [23]. In mammals, a subcellular partition of acetylated tubulin has been found: it is present in axonemes, but not in the singlet microtubules of the manchette [11, 12, 15]. The finding reported here show a similarity between mammalian spermatids and platyhelminth spermatozoa, both having acetylated axonemes and non-acetylated cortical microtubules.

The absence of tubulin in the central core of the 9+"1" axoneme

The 9+"1" axonemal structure is a synapomorphy for the Trepaxonemata [9]. Although the 9+"1" axoneme has been the subject of detailed studies with various methods of electron microscopy [3-6, 14, 29-31], the chemical nature of the central core has not been investigated. This study is, as far as we know, the first attempt to characterize the proteins present in the central core. The central core of the 9+"1" axoneme is not labelled by any of the three monoclonal anti-tubulin antibodies used (anti-alpha-, anti-beta- and anti-alpha-acetylated-tubulin), therefore suggesting the absence of tubulin in the central core. The absence of tubulin in the central core, if confirmed, would underline the uniqueness of the 9+"1" trepaxonematan structure in the Animal Kingdom.

Source : MNHN, Paris

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Source : MNHN, Paris

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Source : MNHN, Paris

Comparative Spermatology of Gastrotricha

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ABSTRACT

Sperm morphology of nine gastrotrich species belonging to the two orders Macrodasyida and Chaetonotida has been examined. By comparison with the limited data in the literature it has been possible to determine a sperm model for macrodasyids: long, corkscrew-shaped acrosome often showing two different portions and containing a striated tube; spring-shaped nucleus surrounding the mitochondria; tail with an axoneme surrounded by a striated cylinder. Within Chaetonotida the spermatozoa are highly diverse: in the family Xenotrichulidae they are characterized by an uncondensed nucleus, two extremely long paraacrosomal bodies, a single mitochondrion and accessory fibres in the tail. The other chaetonotid families show extremely atypical spermatozoa, formed by simple rods of chromatin. There are no evident characters in common among the spermatozoa belonging to the two orders, nor similarities with other aschelminth sperm models.

RÉSUMÉ

Spermatologie comparée des Gastrotricha

La morphologie du spermatozoïde a été étudiée dans neuf espèces de Gastrotriches appartenant aux deux ordres Macrodasyida et Chaetonotida. Grâce à une comparaison avec les données limitées de la littérature, il a été possible de définir un modèle spermatique pour les Macrodasyida: long, avec un acrosome en tire-bouchon qui présente souvent deux portions et contient un tube strié, un noyau hélicoïdal entourant la mitochondrie, une queue avec un axonème entouré par un cylindre strié. Chez les Chaetonotida le spermatozoïde est très diversifié: dans la famille Xenotrichulidae, il est caractérisé par un noyau non condensé, deux corps para-acrosomaux extrêmement longs, une mitochondrie unique et des fibres accessoires dans la queue. Les autres familles de Chaetonotida montrent des spermatozoïdes très atypiques, formés par de simples baguettes de chromatine. Il n'y a pas de caractères spermatologiques en commun unissant les deux ordres, ni de similarités avec les modèles spermatiques des autres Aschelminthes.

Gastrotricha are small (80 µm - 1.5 mm) pseudocoelomate animals living in the sediments of aquatic environments. The order Macrodasyida comprises about 230 species exclusive of marine and brackish interstitial environments, whereas the order Chaetonotida, with about 320 species, is mainly freshwater dwelling, both in interstitial and epibenthic habitats. The two orders have different reproductive modes: Macrodasyida are all amphimictic and hermaphrodite, whereas Chaetonotida are thought to be mainly parthenogenetic with the exception of three hermaphrodite, marine genera [3].

FERRAGUTI, M., & BALSAMO, M., 1995. — Comparative spermatology of Gastrotricha. *In*: JAMIESON, B. G. M., AUSIO, J., & JUSTINE, J.-L. (eds), Advances in Spermatozoal Phylogeny and Taxonomy. *Mém. Mus. natn Hist. nat.*, **166**: 105-117. Paris ISBN: 2-85653-225-X.

Source : MNHN, Paris

TABLE 1. — List of gastrotrich species included in the present review and relevant literature references.

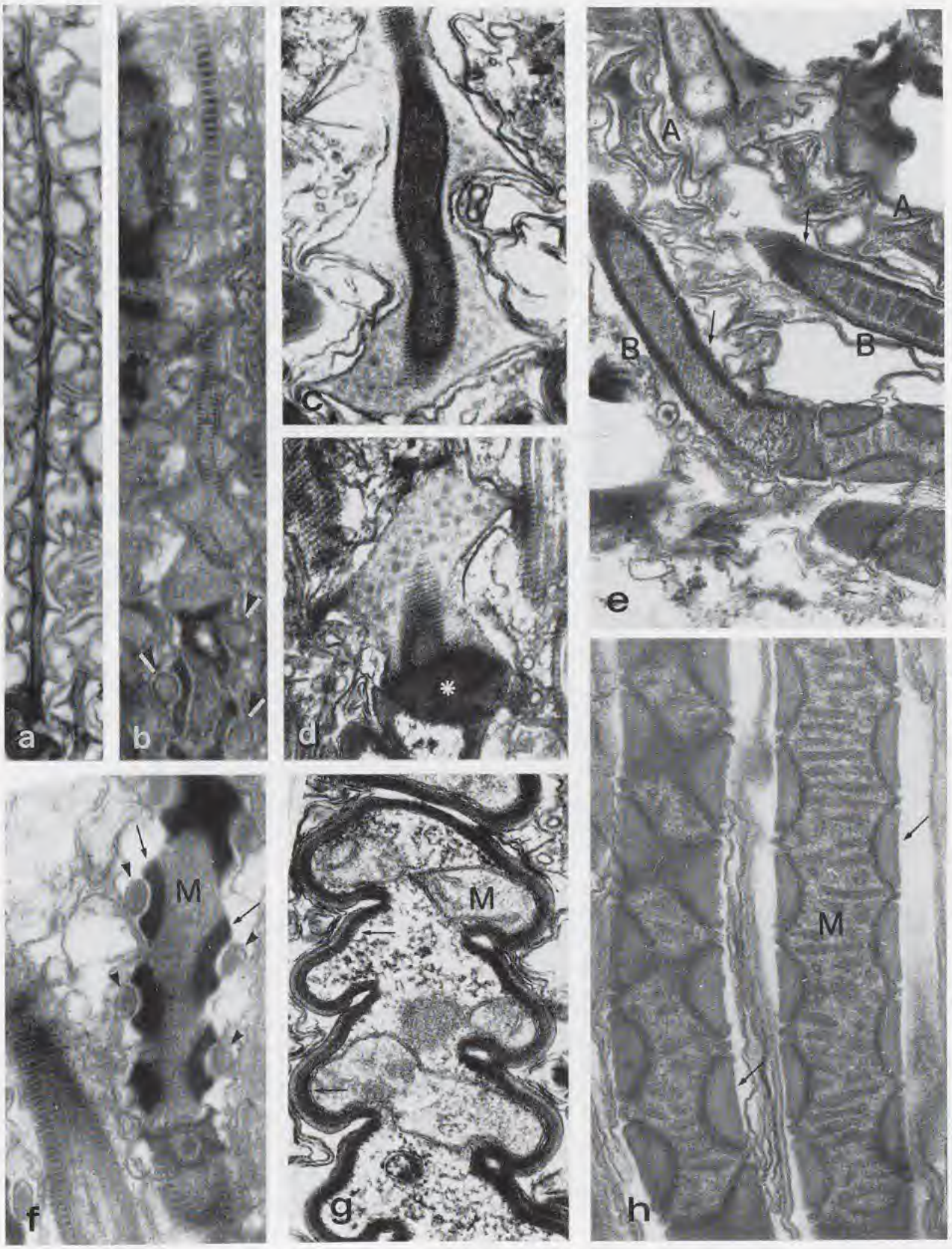
Order	Family	Species	Reference
Macrodasyida	Macrodasyidae	*Macrodasys* sp.	[10]
	Lepidodasyidae	*Mesodasys laticaudatus*	[6]
		Cephalodasys maximus	[8]
	Thaumastodermatidae	*Pseudostomella etrusca*	Present study
		Tetranchyroderma sp. 1	Present study
		Tetranchyroderma sp. 2	Present study
		Tetranchyroderma papii	Present study
		Diplodasys ankeli	Present study
	Turbanellidae	*Turbanella ambronensis*	Present study
		Turbanella cornuta	[12,13]
Chaetonotida	Neodasyidae	*Neodasys* sp.	[11]
	Chaetonotidae	*Chaetonotus maximus*	[3]
		Lepidodermella squamata	[9]
	Xenotrichulidae	*Heteroxenotrichula squamosa*	[7, Present study]
		Xenotrichula intermedia	[7, Present study]
		Xenotrichula punctata	[7, Present study]

Gastrotrich spermatozoa are only imperfectly known (Table 1). In fact only two sperm models of Macrodasyida have been completely described [6, 12, 13], and among Chaetonotida we know with some details only the extremely atypical, perhaps relictual spermatozoon of *Lepidodermella squamata* [9]. Recently some data on the xenotrichulid spermatozoa have been reported [7]. Data on other species are scattered, often restricted to single micrographs, and in some cases inconsistent even within single families. With this poor knowledge, delineation of a generalized sperm model for Gastrotricha is extremely difficult.

In the order Macrodasyida the spermatozoa are filiform cells comprising a sequence of acrosome, nucleus and tail, and devoid of any recognizable midpiece (Fig. 3). Both acrosome and nucleus are corkscrew-shaped: the acrosome shows different regions with complex differentiations [6, 10, 12, 13]; the nucleus is spring-shaped in the lepidodasyid *Mesodasys laticaudatus* [6] and in the turbanellid *Turbanella cornuta* [12, 13], the nuclear spring surrounding the mitochondria in both species. In the lepidodasyd *Cephalodasys maximus*, the nucleus is

FIG. 1. — Ultrastructural features of macrodasyid gastrotrich spermatozoa. **a:** *Tetranchyroderma* sp. 1: anterior part of an acrosome with the striated tube. x 21 000; **b:** *Diplodasys ankeli*: base of the acrosome with the wider portion of the striated tube. A further tubular structure possibly continuous with the acrosome coils around the nucleus (arrowheads). x 40 000; **c, d:** *Pseudostomella etrusca*: main (c) and basal (d) portion of the acrosome: the striated tubule ends in a dense material (asterisk). x 40 000; **e:** *Turbanella ambronensis*: anterior (A) and basal (B) part of the acrosome; the obliquely striated sheath surrounding the basal portion of the acrosome is visible in grazing sections (arrows). x 45 000; **f:** *Diplodasys ankeli*: basal part of the nucleus (arrows) surrounding the mitochondrion (M) and involved by the coiled tubular structure (arrowheads). Note the curious structure at the base of the flagellum (compare with Fig. 2a). x 40 000; **g:** *Pseudostomella etrusca* nuclear region: in this species the chromatin is reduced to a thin lamina (arrows) involving the cytoplasm with some mitochondria (M). x 40 000; **h:** *Turbanella ambronensis*: the nucleus (arrow) is spring-shaped and surrounds a single, long mitochondrion (M). x 60 000.

Source : MNHN, Paris



Source : MNHN, Paris

twisted in the apical portion and spring-shaped, containing the single mitochondrion only in the basal portion [8]. In contrast, in the macrodasyid *Macrodasys* sp. [10] the nucleus is straight and *surrounded* by a mitochondrial helix involving also most of the acrosome. A similar situation was reported also in *Thaumastoderma* sp. [11].

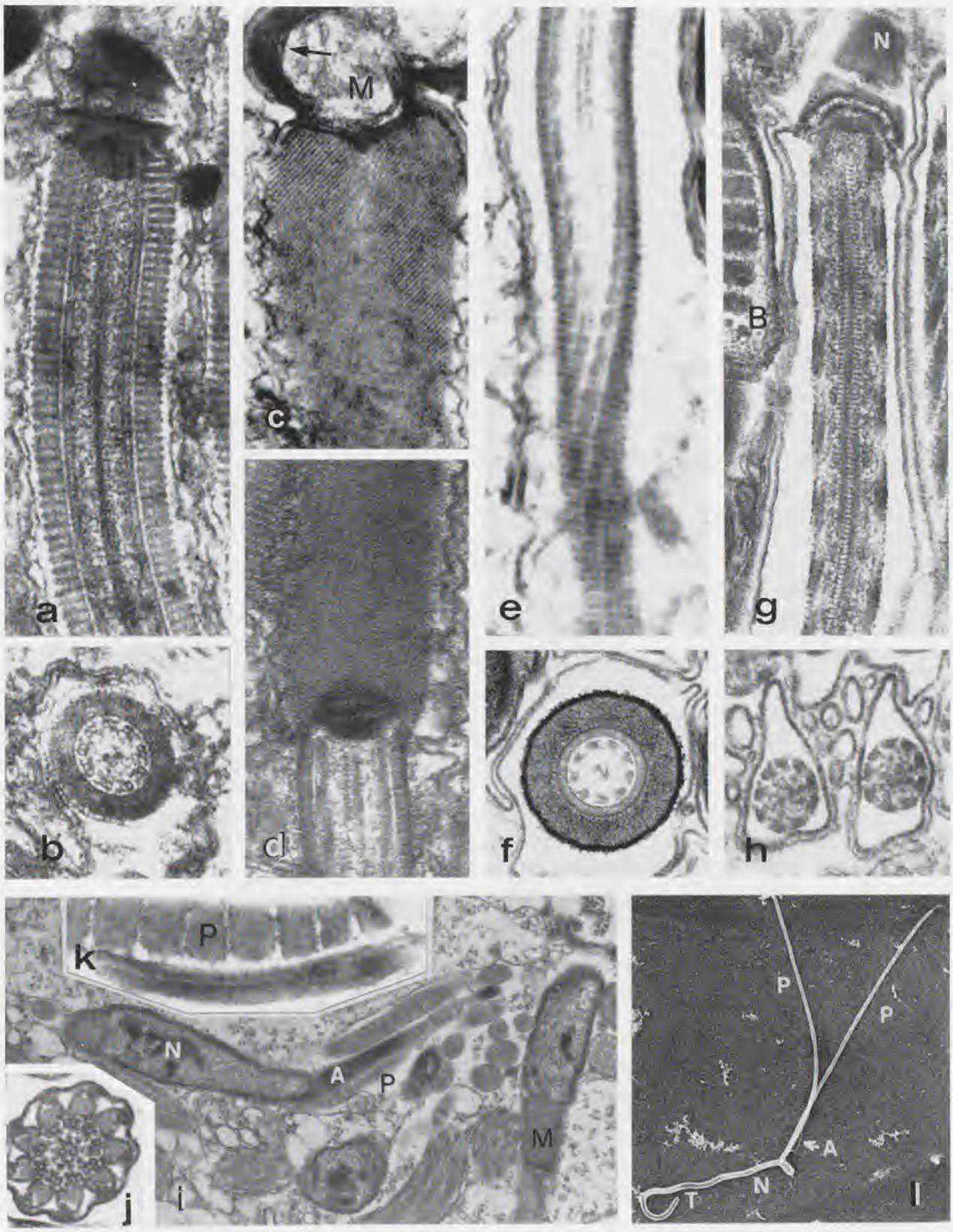
The tail has a 9+2 axoneme enclosed in a striated cylinder in all the macrodasyid species studied so far with the sole exception of *Turbanella cornuta* [12, 13] and, perhaps, *Acanthodasys* sp.[11] (however, the only micrograph published refers to spermatids).

For the order Chaetonotida we only have a single published micrograph of the spermatozoon of the interesting genus *Neodasys* from the monogeneric family Neodasyidae [11]: it shows a very simple and undifferentiated conical acrosome followed by a nucleus perhaps surrounded by a mitochondrial helix. Of the three genera composing the family Xenotrichulidae, *Draculiciteria* is parthenogenetic, whereas *Xenotrichula* and *Heteroxenotrichula* have very peculiar spermatozoa [7] with a small and simple acrosome followed by an uncondensed rectilinear nucleus, a single mitochondrion and a tail with nine peripheral accessory fibres. Furthermore, two of the xenotrichulid species show extremely long paraacrosomal bodies of unknown function. All the other chaetonotid families, comprising hundreds of species, are parthenogenetic. However, hermaphroditic individuals of most genera have been discovered both in culture and in nature and production of the spermatozoa during the long post-parthenogenetic phase of the life cycle has been demonstrated in at least four genera of the family Chaetonotidae [3, 9]. Low numbers of these spermatozoa are usually present, with four different morphologies. In *Lepidodermella squamata* [9] and *Chaetonotus maximus* [3] the spermatozoa are only rods of condensed chromatin surrounded by plasma membrane without organelles of any kind.

Thus, comparative spermatology of Gastrotricha is still in its infancy. For many years we have undertaken a study of selected gastrotrich species to extend our knowledge of their spermatozoa. However, the technical problems in working with such material (extremely small size of these animals; difficult fixation; problems with embedding and orientation of specimens within the resin; failure of attempts to rear macrodasyids in the laboratory) have impeded our project. Knowing the importance of the spermatozoa as systematic characters in comparisons within and among taxa, in this paper we will present some work in progress on previously undescribed gastrotrich species. We will focus our attention on the family Thaumastodermatidae, with *Pseudostomella etrusca*, three species of *Tetranchyroderma*, and *Diplodasys ankeli*; we will describe a previously unknown spermatozoon from the Turbanellidae, *Turbanella ambronensis*; finally we will extend the description of the sperm of the three Xenotrichulidae [7]. We will present some ultrastructural details of the species described and three-dimensional reconstructions of four gastrotrich spermatozoa.

FIG. 2. — Ultrastructure of the spermatozoa of gastrotrichs. **a-h:** macrodasyids; **i-l:** chaetonotids. **a, b:** *Diplodasys ankeli*: longitudinal (a) and cross (b) section of a tail to show the structure of the striated cylinder. x 60 000; **c, d:** *Pseudostomella etrusca*: longitudinal sections of the crystalline structure situated between the nucleus (arrow) and the tail. M, mitochondrion. x 60 000. **e:** *Tetranchyroderma* sp. 1: longitudinal, sagittal (top) and tangential (bottom) section of the tail showing the structure of the striated cylinder. x 60 000; **f:** *Tetranchyroderma* sp. 2: cross section of a tail showing the double striated cylinder. x 60 000; **g, h:** *Turbanella ambronensis*: longitudinal (g) and cross (h) sections of a tail showing no striated cylinder. N, nucleus; B, basal portion of the acrosome. x 60 000; **i, j, k:** *Xenotrichula punctata*; **i:** low power view of some spermatozoa: N, nucleus; A, acrosome; P, paraacrosomal body; M, mitochondria. x 20 000; **j:** cross section of a tail. Note the prominent accessory fibres. x 60 000; **k:** longitudinal section of an acrosome (bottom) and a paraacrosomal body (P). x 60 000; **l:** *Heteroxenotrichula squamosa*: S.E.M. view of a whole spermatozoon. P, paraacrosomal body; A, acrosome; N, nucleus; T, tail. x 2 800.

Source : MNHN, Paris



Source : MNHN, Paris

MATERIAL AND METHODS

Gastrotrichs were extracted from sandy sediments collected in some localities of the Tyrrhenian and Adriatic coasts of Italy by decantation and narcotization with 7% $MgCl_2$. Observations on living specimens were carried out by means of a Leitz Dialux microscope equipped with Nomarski optics and phase contrast. Moving spermatozoa within the testes, and after isolation, were recorded on videotape.

Transmission Electron Microscopy (TEM). Specimens were fixed in a 0.1 M phosphate buffered (pH 7.3) mixture of paraformaldehyde, glutaraldehyde and picric acid with addition of sucrose (SPAF) [5], postfixed in 2% aqueous osmium tetroxide, washed in 0.1 M cacodylate buffer, dehydrated in a graded acetone series, pre-stained *en bloc* with uranyl acetate in 70% acetone and embedded in araldite. Sections were cut with an Ultrotome Nova LKB, triple stained following DADDOW [4], carbon coated and observed under a JEOL 100 XS electron microscope.

Scanning Electron Microscopy (SEM). The spermatozoa were isolated from living animals and fixed with the same mixture used for TEM, dehydrated in a graded ethanol series, critical point dried with CO_2, coated with gold-palladium and observed with a Philips XL40 or a Cambridge Stereoscan 250Mk2.

OBSERVATIONS

Macrodasyida

Family Thaumastodermatidae. The spermatozoa of *Pseudostomella etrusca* (Fig. 4) show a complex, corkscrew-shaped acrosome 5-7 µm long containing a matrix in which a tubular striated structure is immersed (Fig. 1c, d). The striated tube ends basally in an area of dense material which forms the base of the acrosome (Fig. 1 d).The nucleus follows with the typical shape of a hollow corkscrew (Fig. 1g), about 20 µm long. The chromatin is visible as a 0.1 µm thick external sheath whereas the inner cavity contains some cytoplasm with numerous mitochondria (Figs 1g, 2c). Between the nucleus and the tail there is a crystalline structure, about 1.6 µm long and 0.5 µm in diameter (periodicity of about 13 nm) (Fig. 2c, d). The tail consists of a 9+2 axoneme surrounded by a striated cylinder formed by a pile of rings about 13 nm thick connected by thin threads (Fig. 2 d).

We have examined three species of the genus *Tetranchyroderma*: an unidentified species from Tyrrhenian sea (*T.* sp. 1); an undescribed species from the Maldive archipelago (*T.* sp. 2); and *T. papii* from the Tyrrhenian sea. The acrosome of *T.* sp.1 and *T. papii* show an apical filament followed by a corkscrew region for a total length of approximately 8 µm (*T. papii*). The acrosome axis is a striated tube (Fig. 1 a) ending in a basal mass of dense material touching the nucleus (*T.* sp. 1). The ribbon-like nucleus coils around the single long mitochondrion in *T.* sp. 1. An additional tubular structure runs for two gyres around the nuclear apex in *T.* sp. 1 whereas it wraps around the whole nucleus in *T. papii*. The tubular structure continues the pitch of the acrosome, and may also be structurally continuous with it. A crystalline structure similar to the one already described in *P. etrusca* is visible in *T. papii* and in *T.* sp. 2, but is absent in *T.* sp. 1. The tail axis is a 9+2 axoneme surrounded by a striated cylinder formed by a pile of disks 15 nm thick connected by thin threads (*T.* sp.1 and *T.* sp 2) (Fig. 2e). In *T.* sp. 2 a second external layer is present, similar in texture to the crystalline structure at the base of the tail. This additional sheath is thicker (up to 120 nm) at the base of the flagellum (Fig. 2f), then flattens to a size comparable to that of the striated cylinder. Images of "empty" tails, in which the striated cylinder is present but there is no axoneme, suggest that the axoneme is shorter than the striated cylinder.

Diplodasys ankeli has a long, thin, corkscrew-shaped acrosome (Fig. 1b) in which two regions can be recognized: the anterior one, 0.15 µm in diameter with a helical pitch of about 0.26 µm contains a twisted column with alternate dark and pale cross bands, whereas the basal portion, about 1 µm long, contains a twisted striated tube. The following nucleus is a spring of condensed chromatin surrounding the single mitochondrion (Fig. 1b, f). A tubular structure possibly continuous with the acrosome coils around the nucleus for its whole length (Fig. 1b, f). The tail has a 9+2 axoneme enclosed by a striated cylinder formed by a pile of discs (Fig. 2 a, b).

Source : MNHN, Paris

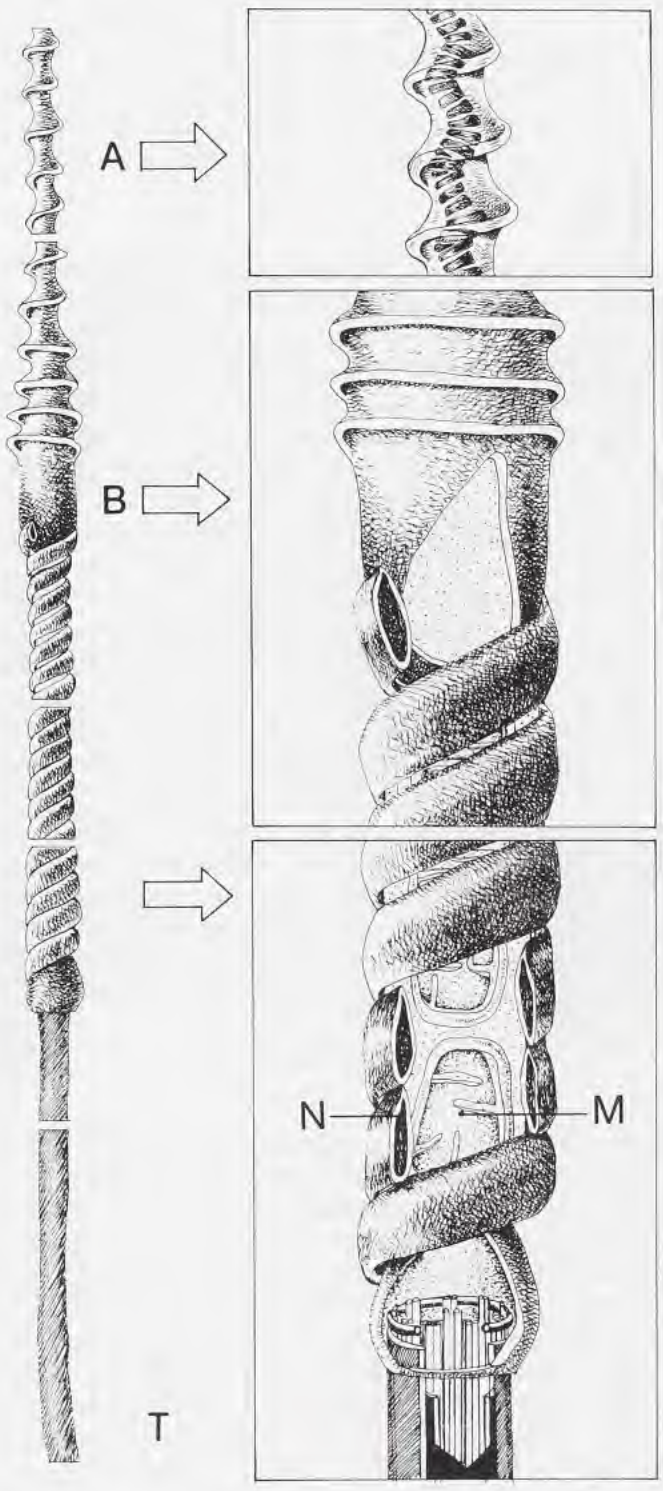


FIG. 3. — Schematic drawing of *Mesodasys laticaudatus* spermatozoon. A, anterior portion of the acrosome: the striated column is visible in the detail (right); B, basal portion of the acrosome. A detail is visible at right; N, nucleus; M, mitochondria; T, tail.

Source : MNHN, Paris

Family Turbanellidae. The acrosome of *Turbanella ambronensis* has two different regions (Figs 1e, 5): the anterior one is thin (0.2 µm), corkscrew-shaped and contains a twisted axis made of alternating dense and transparent bands; the basal portion is long and rectilinear and shows an obliquely striated sheath surrounding the acrosome content (Fig. 1e). This is formed by a pile of thick (60-80 nm) disks immersed in a transparent matrix. The nucleus is ribbon-like, D-shaped in cross section (Fig. 1h), and coiled to form a spring inside which a single, long mitochondrion is located. The tail has a normal 9+2 axoneme but no striated cylinder (Fig. 2g, h).

Chaetonotida

Family Xenotrichulidae. We have studied the spermatozoa of three xenotrichulid species: *Heteroxenotrichula squamosa*, *Xenotrichula intermedia*, and *X. punctata*. The spermatozoa of these species have many characters in common, thus they will be described together. The xenotrichulid spermatozoa are characterized by a sequence of acrosome, nucleus, mitochondrion and tail (Fig. 6). The acrosome is a small structure (3.8 µm long with a diameter of 0.1 µm in *H. squamosa* and 2.9 µm long in *X. punctata*) containing an acrosome vesicle and a large amount of periacrosomal material (Fig. 2k, i, l). Two projecting structures parallel to the acrosome are inserted at the anterior extremity of the nuclear region in *X. punctata* and *H. squamosa* (Fig. 2i, l) where they are 20 µm long with a diameter of 0.2 µm . These structures are not surrounded by a plasma membrane and consist of electron dense disks 0.1 µm thick connected by thin threads (Fig. 2k); we have called them paraacrosomal bodies [7]. In the insertion area the plasma membrane of the spermatozoon is uninterrupted [7], suggesting that the paraacrosomal bodies are external to the cell. *X. intermedia* is an exception in having no acrosome nor paracrosomal bodies. The nucleus is characterized by a scarcely condensed chromatin in the three species examined. Only in *X. punctata* scattered dense globules of condensed chromatin were observed (Fig. 2i), whereas in the other species examined the chromatin resembles that of a somatic cell. A single, conventional mitochondrion of variable length follows the nucleus (Figs 2i, 6). A short tail is the last portion of the xenotrichulid spermatozoon. In its principal tract the axoneme is surrounded by nine large electron dense accessory fibres which are external to and in correspondence with each axonemal doublet (Fig. 2j). They have a somewhat striated appearance in grazing longitudinal sections whereas in cross section they appear pear-shaped with the thinner extremity directed towards the cell membrane and the larger one towards the doublets. The last portion of the *X. intermedia* spermatozoon shows a short tract of the axoneme devoid of accessory fibres.

DISCUSSION

Macrodasyida

The spermatozoa of members of four macrodasyid families have been examined up to now: Lepidodasyidae, Macrodasyidae, Thaumastodermatidae, and Turbanellidae (Table 1). The spermatozoa of Lepidodasyidae, Thaumastodermatidae (with the reported exception of Thaumastoderma [11]) and Turbanellidae have a common general architecture: the acrosome is at least in part corkscrew-shaped and contains various materials with different aspects; the nucleus is always, at least in part, spring-shaped and contains some cytoplasm and one or more mitochondria; a true basal body is lacking. Interspecific variations affect the acrosome which may be divided in two portions, the anterior one containing a twisted axis made of dense and pale disks, as in *Mesodasys laticaudatus* [6], *D. ankeli* and *T. ambronensis*. The basal portion of the acrosome may be rectilinear (*T. ambronensis, M. laticaudatus*) and contain a hollow striated tube, as in *D. ankeli* and *Cephalodasys maximus* [8]. A twisted hollow striated tube may run for the whole length of the acrosome, as in *P. etrusca* and *T.* sp.1 where a mass of dense material is also present at the base of the acrosome. *Cephalodasys maximus* has an apical acrosome vesicle protruding anteriorly [8]. If this report were confirmed, the real nature of the long structure

Source : MNHN, Paris

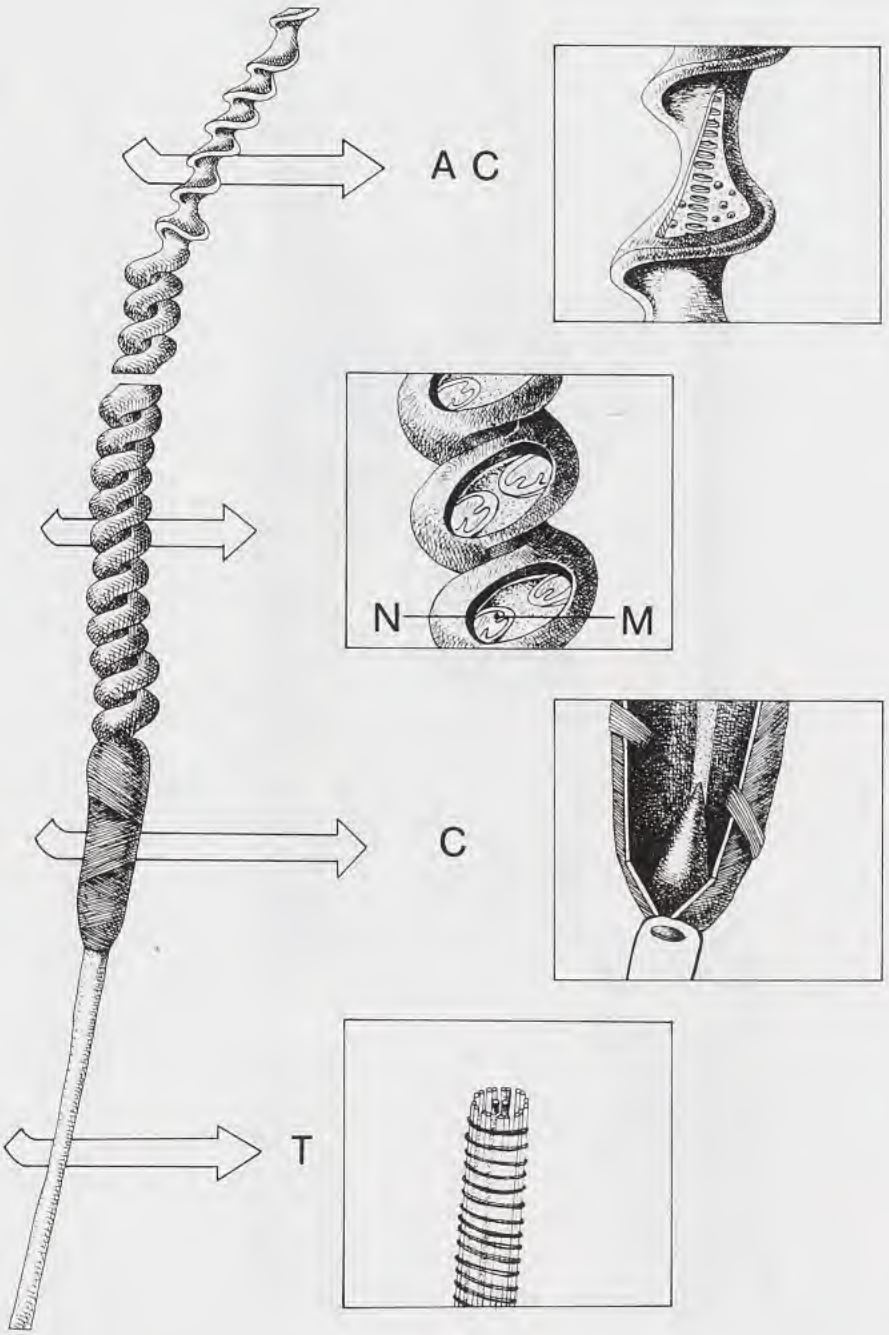


FIG. 4. — Schematic drawing of *Pseudostomella etrusca* spermatozoon. AC, acrosome: in the enlarged detail (right) the inner structure of the acrosome is visible; N, nucleus; M, mitochondria; C, crystalline structure; T, tail.

Source : MNHN, Paris

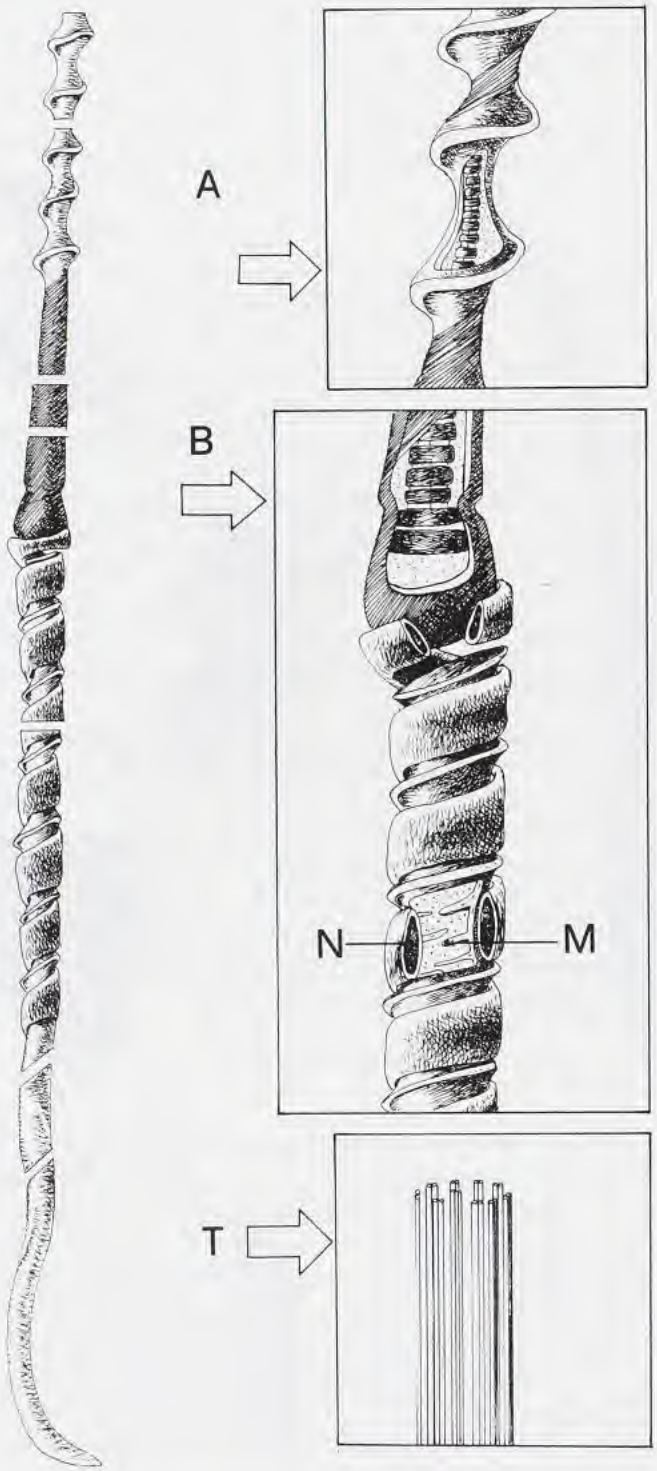


FIG. 5. — Schematic drawing of *Turbanella ambronensis* spermatozoon. A, anterior portion of the acrosome with the striated column inside (detail at right); B, basal portion of the acrosome with the obliquely striated sheath; N, nucleus; M, mitochondria; T, tail.

Source : MNHN, Paris

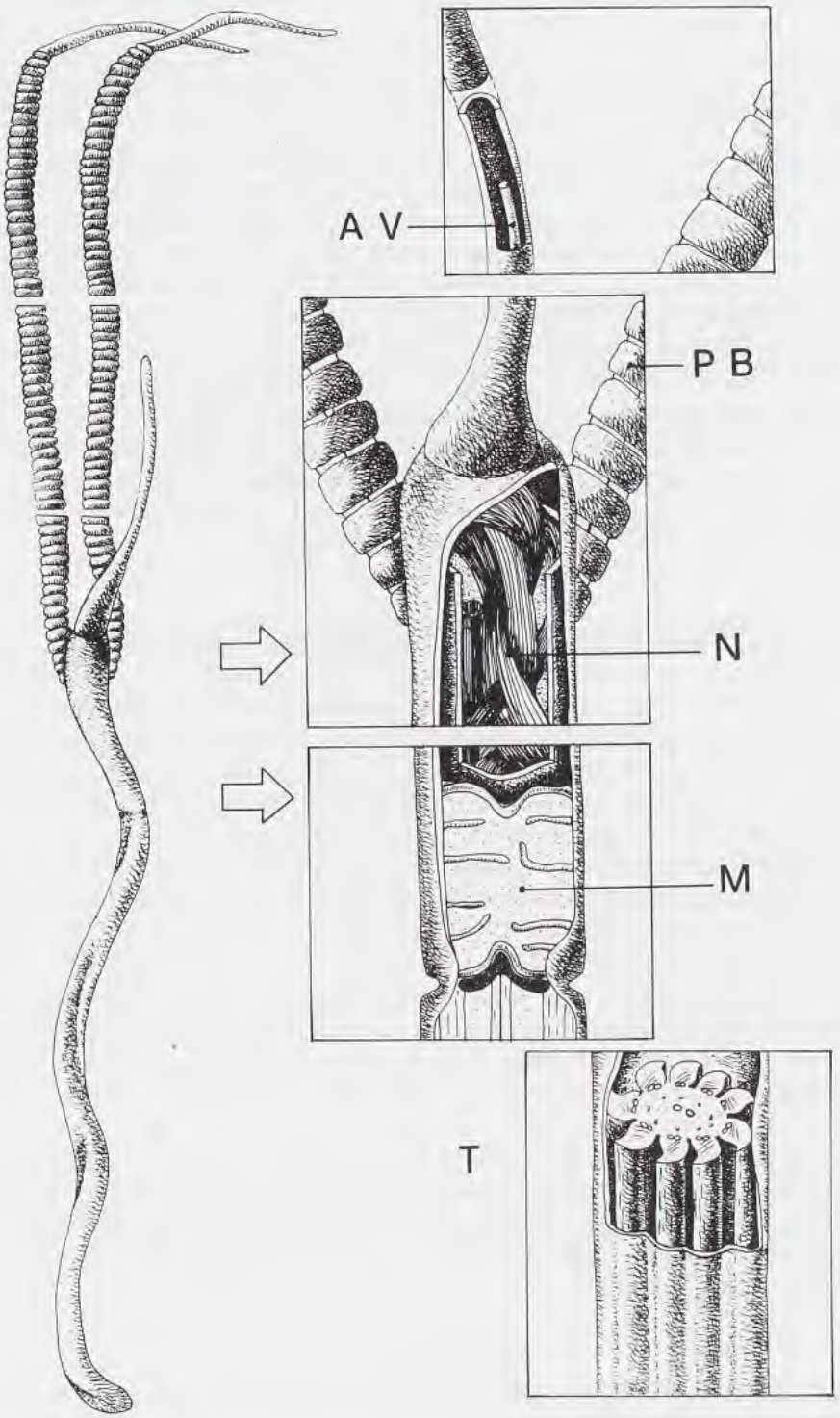


FIG. 6. — Schematic drawing of *Heteroxenotrichula squamosa* spermatozoon. AV, acrosome vesicle; PB, paraacrosomal body; N, nucleus; M, mitochondrion; T, tail.

Source : MNHN, Paris

defined as an "acrosome" in this species would remain to be established. The acrosome of Macrodasyidae briefly described by RUPPERT [10] may share some features with that of the other Macrodasyida. In *Macrodasys* sp., in fact, the acrosome has two portions: the basal one with a "hollow central acrosomal filament" and the anterior one with the filament widening and twisting. A marked difference lies in the mitochondrial spiral winding around the acrosome in *Macrodasys*. The sperm nucleus of all the species, with the exception of *Macrodasys* sp. and, reportedly, of *Thaumastoderma* sp.[11] is a spiral surrounding the cytoplasm. Variations concern the number and morphology of mitochondria and the presence of the additional tubular structure coiled around the nucleus. This additional tubular structure runs along the whole nucleus in some species but is restricted to the nuclear apex in others and may be continuous with the acrosome. In *Cephalodasys maximus*, part of the acrosome is reported to involve the first gyre of the nuclear helix [8]. Mitochondria may retain their conventional aspect, as in *P. etrusca*, or be fused in a single, twisted column-shaped, mitochondrion filling the whole space delimited by the nuclear spiral, as in *Turbanella* (this study and [12, 13]) and *Cephalodasys* [8].

The tail shows a characteristic sheath called the striated cylinder [6], or the spiralled band [8], enclosing the 9+2 axoneme in all families except the Turbanellidae. In *Cephalodasys maximus* in addition to the striated cylinder there are nine dense strings [8] (probably accessory fibres), one corresponding with each flagellar doublet. In this last species, a manchette of oblique microtubules is reported beneath the flagellar plasma membrane.

Chaetonotida

The spermatozoa of Chaetonotida show a much greater interfamilial diversity with respect to Macrodasyida. The sperm models of *Neodasys* [11], Xenotrichulidae ([7] and this chapter), and Chaetonotidae[3, 9] studied so far are different from one another and bear no resemblance to those of Macrodasyida. In particular the spermatozoa of Xenotrichulidae are unique not only among Gastrotricha , but in the entire animal kingdom in the large paraacrosomal bodies (not present, for unknown reasons, in one of the three species examined). The other outstanding characters of the xenotrichulid spermatozoa are: the presence of a single, untransformed mitochondrion placed between the nucleus and the tail and of the large accessory fibres of the flagellum. The shape and position of the single mitochondrion is a curious convergence with leech spermatozoa [14], whereas the accessory fibres are an independent invention of many evolutionary lineages with internal fertilization [2].

Present knowledge of gastrotrich sperm morphology does not allow the delineation of a general sperm model for Gastrotricha. Furthermore, if some characters are common among most Macrodasyida (complex corkscrew-shaped acrosome, spring-shaped nucleus containing mitochondria, striated cylinder in the tail), the three Chaetonotida models known so far present a puzzling diversity.

Comparisons with the spermatozoa of other aschelminthes are also deceiving. The general architecture of gastrotrich spermatozoa seems unique among pseudocoelomates. We may note that if the tubular spiral structure involving the nucleus of some Thaumastodermatidae is proved to be continuous with the acrosome, this could be an important resemblance with the spermatozoon of the priapulid *Tubiluchus corallicola* [1].

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Source : MNHN, Paris

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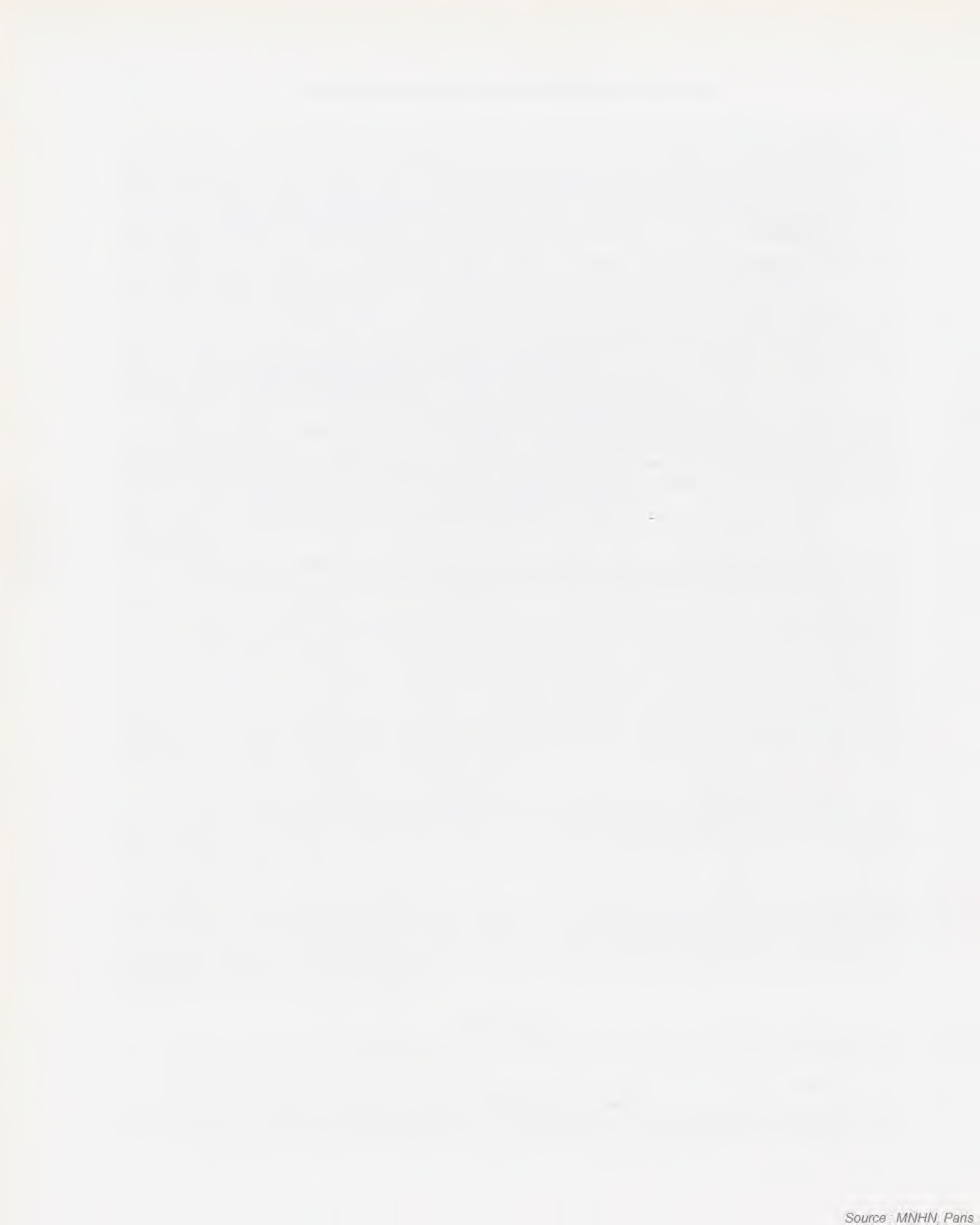
Source : MNHN, Paris



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Centrioles with Ten Singlets in Spermatozoa of the Parasitic Nematode *Heligmosomoides polygyrus*

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ABSTRACT

The spermatozoon of *Heligmosomoides polygyrus* is elongate and aflagellate. The nucleus, in a posterior position, is arrowhead-shaped and has an anterior fossa. Two centrioles, which are the only microtubular organelles of the mature spermatozoon, are mutually perpendicular in the nuclear fossa. These two centrioles are made up of 10 singlets, closely arranged, forming an ellipse 140 x 160 nm. This is the first description of a centriole with 10 singlets. Immunocytochemistry demonstrates that spermatids have a transitory system of microtubules converging toward the centrioles. Spermatozoal centrioles may provide additional character for the understanding of nematode phylogeny.

RÉSUMÉ

Centrioles à dix singulets dans les spermatozoïdes du nématode parasite *Heligmosomoides polygyrus*

Le spermatozoïde de *Heligmosomoides polygyrus* est allongé et aflagellé. Le noyau, placé postérieurement, est en forme de pointe de flèche et présente une fossette à sa partie antérieure. Deux centrioles, qui sont les seuls organites composés de microtubules dans le spermatozoïde mûr, sont disposés perpendiculairement dans la fossette nucléaire. Ces deux centrioles sont composés de 10 singulets juxtaposés, formant une ellipse de 140 x 160 nm. Ceci est la première description d'un centriole à 10 singulets. L'immunocytochimie montre que les spermatides possèdent un système transitoire de microtubules convergeant vers les centrioles. Les centrioles des spermatozoïdes peuvent fournir de nouveaux caractères pour la compréhension de la phylogénie des Nématodes.

The spermatozoa of the Nematoda are all aflagellate. Axonemes are always absent but microtubules are sometimes present during spermiogenesis. In mature spermatozoa, microtubules have been described only in a few species [6]. Table 1 is a list of species in which the spermatozoon has been described by electron microscopy.

In *Heligmosomoides polygyrus*, a parasitic nematode often used in laboratory experiments, we found an outstanding centriolar structure, which is described here. The spermatozoon has been described previously in this species [81, 84], but centriolar structure was not addressed.

In addition, the present paper gives some information on the fate of the microtubular system of spermatids, observed by mean of immunocytochemistry of tubulin.

MANSIR, A., & JUSTINE, J.-L., 1995. — Centrioles with ten singlets in spermatozoa of the parasitic nematode *Heligmosomoides polygyrus*. *In*: JAMIESON, B. G. M., AUSIO, J., & JUSTINE, J.-L. (eds), Advances in Spermatozoal Phylogeny and Taxonomy. *Mém. Mus. natn. Hist. nat.*, **166** : 119-128. Paris ISBN : 2-85653-225-X.

Source : MNHN, Paris

MATERIALS AND METHODS

Material. Samples of *Heligmosomoides polygyrus* (Dujardin, 1845) were obtained from laboratory white mice, strain CD1. This species is sometimes named *Nematospiroides dubius* but this is a junior synonym [20, 21]. The strain used here belongs to the subspecies *Heligmosomoides polygyrus bakeri* Durette-Desset, Kinsella & Forrester, 1972 [19], originally collected from *Peromyscus maniculatus* in North America and transferred to laboratory mice, strain CD1, in which it has been maintained for more than twenty years [46]. Adults were collected two to four weeks after oral infestation with 300 larvae. Males were isolated under a binocular microscope and kept for a few hours in salt water (NaCl 9‰).

Electron microscopy. Living specimens were placed in cold (4 °C) fixative, the head being immediately cut to allow the fixative to penetrate into the body. Specimens were fixed for 1 h in 2% glutaraldehyde in a buffer solution of 0.1 M sodium cacodylate at pH 7.2 at 4°C. After rinsing in the same buffer, the worms were postfixed for 1 h in 1% osmium tetroxide in the same buffer, dehydrated in ethanol and propylene oxide, and embedded in SPURR's medium [69]. Ultrathin sections were contrasted with DADDOW's method [15], and observed with a Hitachi H600 electron microscope.

Immunocytochemistry. Germ cells were obtained by dissecting each male in a drop of PBS (phosphate buffer saline, Sigma) on a pit slide previously washed with alcohol and acetone. The genital system was gently pressed with thin needles to release germ cells. Slides were kept in a humid chamber for 1-2 hours to allow cells to sink and adhere to the slide. The PBS was then removed and replaced by a drop of 3.7% formaldehyde in PBS for fixation. After 15 min the pit was rinsed with PBS (3 x 5 min). Cells were then permeabilized in 0.1% Triton X-100 in PBS and rinsed (PBS, 3 x 5 min). Non-specific antigens were blocked with 2% Bovine Serum Albumin (Sigma) in PBS (BSA-PBS) for 45-90 min at room temperature. Without intermediary washing, a monoclonal anti-tubulin antibody (anti-alpha-tubulin, clone DM 1A, Sigma, or anti-beta-tubulin, clone TUB 2.1, Sigma or anti-acetylated-tubulin, clone 6-11B-1, Sigma) diluted at 1/200 in BSA-PBS was applied for 40 min at room temperature. After rinsing (PBS 3 x 5 min) the FITC-conjugated antibody (Goat anti-mouse, Nordic, 1/40 in PBS) was applied for 40 min at room temperature. After a final wash (PBS 3 x 5 min), mounting was done in Citifluor (Citifluor Ltd, London, UK) and slides were sealed with nail enamel. Controls were done by omitting the first antibody or by using a non-relevant mouse antibody. Observations were made with a Nikon Optiphot epifluorescence microscope equipped with mercury lamp and a single band Nikon filter for FITC channel (B-2A).

RESULTS

Electron microscopy of mature spermatozoa (Fig. 1)

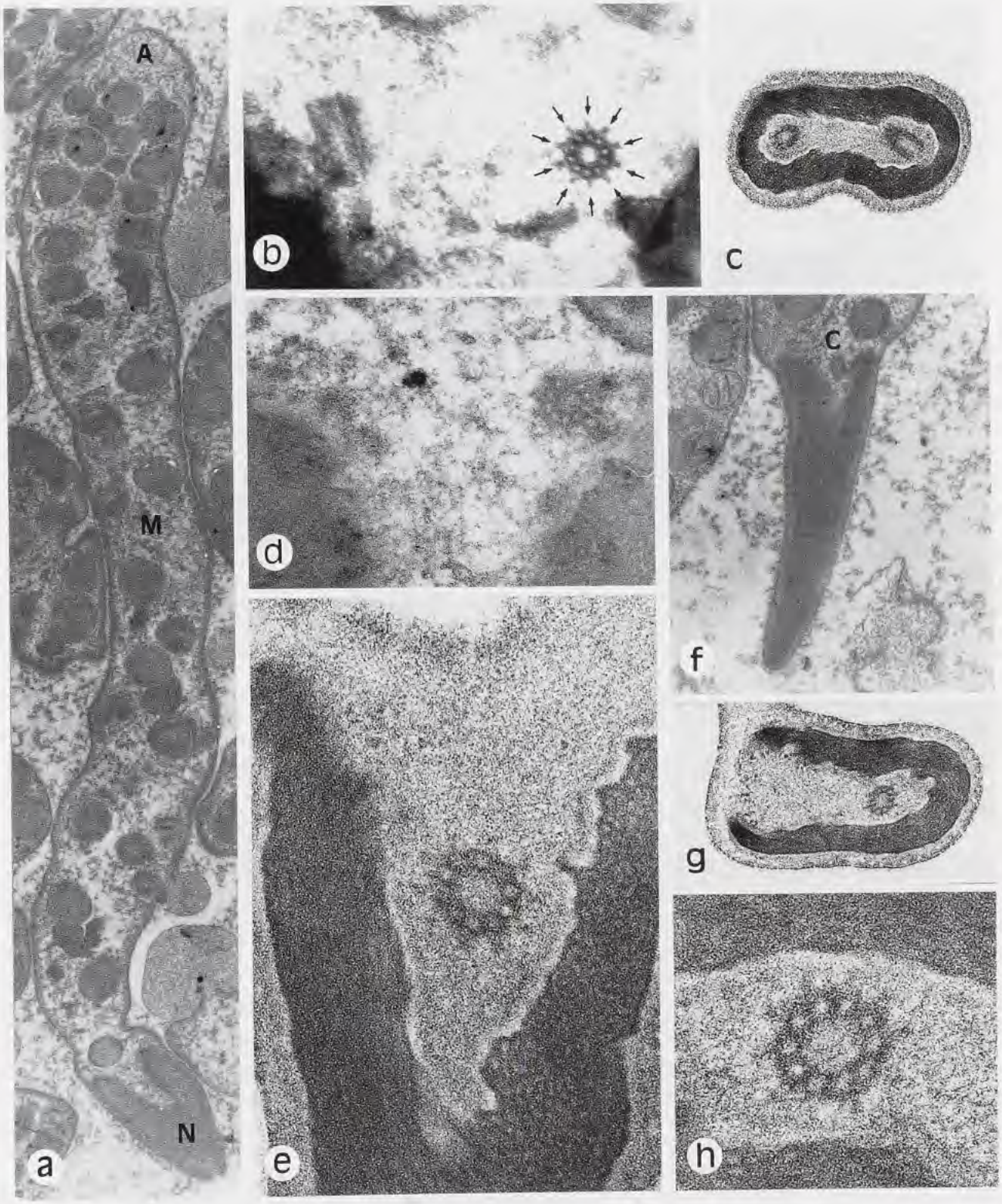
The spermatozoon of *Heligmosomoides polygyrus*, observed in the testis, is an elongate and aflagellate cell (Fig. 1a), about 17 µm in length and 3 µm in width. It comprises three region: 1. The anterior region, opposite the nucleus, is devoid of organelles and contains a fibrillar cytoplasm. It is known that this region produces pseudopods in activated spermatozoa and is functionally anterior [81, 84]. 2. The median region contains round mitochondria and membranous organelles, which are similar to those described in other nematode spermatozoa. 3. The posterior region contains an arrowhead-shaped nucleus, with highly condensed chromatin. The nuclear envelope is absent, as in most nematodes. The anterior fossa of the nucleus contains two centrioles, which are the only microtubular elements of the mature spermatozoon.

The two centrioles have different orientations (Fig. 1b, c). One is aligned or slightly oblique along the longitudinal axis of the spermatozoon, and will be here termed longitudinal. The other centriole is perpendicular to the other and will be termed perpendicular.

The perpendicular centriole is made up of 10 singlets (Fig. 1b-e). Each singlet is in contact with its two neighbours. The singlets are not arranged in a perfect circle, but in an ellipse (140 x 160 nm). Ten dense peripheral elements are visible at the periphery of the centriole: relatively

FIG. 1. — Electron microscopy of *Heligmosomoides polygyrus* spermatozoon, showing centrioles with 10 singlets. **a:** Longitudinal section of spermatozoon, showing anterior region (A), median region (M) and posterior region with nucleus (N). **b, c:** Sections showing the two centrioles in the nuclear fossa. **b:** Longitudinal, relatively thick, section of spermatozoon. The perpendicular centriole (right) shows 10 peripheral spokes (arrows); **c:** Transverse section. **d, e:** Perpendicular centriole in longitudinal section of the spermatozoon. Note the 10 peripheral dots. **f-h:** Longitudinal centriole. **f:** Longitudinal section of nucleus, showing centriole in nuclear fossa. **g, h:** Transverse section of spermatozoon, showing longitudinal centriole with 10 singlets and peripheral dots. In e and h, note elliptic shape of centriole and singlets closely associated. a, x 12 000; b, d, x 45 000; c, x 30 000; e, x 100 000; f, x 15 000; g, x 34 000; h, x 150 000.

Source : MNHN, Paris



Source : MNHN, Paris

TABLE 1. — Spermatozoa of nematodes described by electron microscopy. Classification according to INGLIS (1983) [32]. Many orders of the Nematoda have not been studied for spermatozoal ultrastructure and thus are not cited here.

Class Rhabditia	
Subclass Rhabditia	
Order Rhabditida	
Caenorhabditis elegans	[48, 51, 57-59, 64, 77, 78, 80]
Cephalobus cf *quinilineatus*	[6]
Heterorhabditis bacteriophora	[28, 52]
Neoaplectana intermedia	[29]
Rhabditis pellio	[7]
Order Oxyurida	
Aspiculuris tetraptera	[41]
Order Strongylida	
Ancylostoma caninum	[24, 71-73]
Angiostrongylus cantonensis	[24]
Angiostrongylus cantonensis	[24]
Heligmosomoides polygyrus	[81, 84] This study
Metastrongylus apri	[14]
Nematodirus battus	[44]
Nippostrongylus brasiliensis	[33, 82, 83]
Protostrongylus rufescens	[4]
Subclass Diplogasteria	
Order Diplogasterina	
Panagrellus silusiae	[50]
Order Ascaridida	
Ascaris megalocephala	[22]
Ascaris suum	[1-3, 9, 12, 13, 23, 24, 26, 38, 56, 63]
Heterakis gallinarum	[40]
Polydelphis sp.	[24]
Pseudoterranova decipiens	[42]
Rhigonema madecassum	[76]
Order Spirurida	
Brugia pahangi	[9]
Dipetalonema dessetae	[43]
Dipetalonema setariosum	[45]
Dipetalonema viteae	[25, 45]
Dirofilaria immitis	[24, 45]
Dirofilaria immitis	[24]
Gnathostoma sp.	[24]
Litosomoides carinii	[45]
Loa loa	[79]
Physaloptera sp.	[24]
Subclass Tylenchia	
Order Tylenchida	
Aphelenchoides blastophthorus	[65]
Ekphymatodera thomasoni	[11]
Heterodera (Glob.) rostochiensis	[67]
Heterodera (Globodera) virginiae	[67]
Heterodera (Heterodera) avenae	[67]
Heterodera (Heterodera) schachtii	[67]
Meloidodera floridensis	[10]
Meloidogyne acronea	[66]
Meloidogyne arenaria	[66]
Meloidogyne graminicola	[66]
Meloidogyne hapla	[27, 66]
Meloidogyne incognita	[66]
Meloidogyne oryzae	[66]
Tylenchulus semipenetrans	[6]
Verutus volvingentis	[10]
Class Enoplea	
Subclass Enoplia	
Order Enoplida	
Deontostoma californicum	[85]
Enoplus anisospiculus	[87]
Enoplus demani	[87]
Mesacanthion hirsutum	[6]
Subclass Dorylaimia	
Order Dorylaimida	
Xiphinema diversicaudatum	[6]
Xiphinema theresiae	[39, 74]
Order Dioctophymatida	
Dioctophyma renale	[24]
Order Mermithida	
Gastromermis sp.	[53]
Order Mononchida	
Mylonchulus nainitalensis	[6]
Order Trichurida	
Capillaria hepatica	[47]
Trichinella nativa	[30, 31]
Trichinella pseudospiralis	[31]
Trichinella spiralis	[30, 31, 68, 70]
Trichuris muris	[34, 35]
Class Chromadorea	
Subclass Monohysteria	
Order Monhysterida	
Sphaerolaimus hirsutus	[37, 49]

thick sections (Fig. 1b) show a triangular spoke 30 nm long extending from the peripheral limit of two singlets, and thin sections (Fig. 1d, e) show a dot located at a distance of 30 nm, and located at the level of this limit. The centre of the centriole shows no dense structure.

The longitudinal centriole (Fig. 1b, c, f-h) is also made up of 10 singlets and has the 10 peripheral elements. Lengths ranging from 200 nm (Fig. 1f) to 330 nm (Fig. 1b) have been found, possibly reflecting a change of length during the maturation of the spermatozoon.

Source : MNHN, Paris

Immunocytochemistry of tubulin in spermatids and spermatozoa (Fig. 2)

The immunolocalization of tubulin in mature spermatozoa gives absolutely no reaction, and thus is not illustrated here. However, spermatids show the presence of tubulin. Early elongate spermatids (Fig. 2a) show a heavy labelling of tubulin around the nucleus and in the elongating part of the cytoplasm. The most intense labelling is found in a triangular region located inside the anterior fossa of the nucleus. More advanced spermatids (Fig. 2b) show labelling only in the fossa and in a longitudinal central core in the cytoplasm. Finally, only a few parallel longitudinal microtubules are visible, labelling of which is most intense in the fossa (Fig. 2c). Mature spermatids (Fig. 2d) show no labelling.

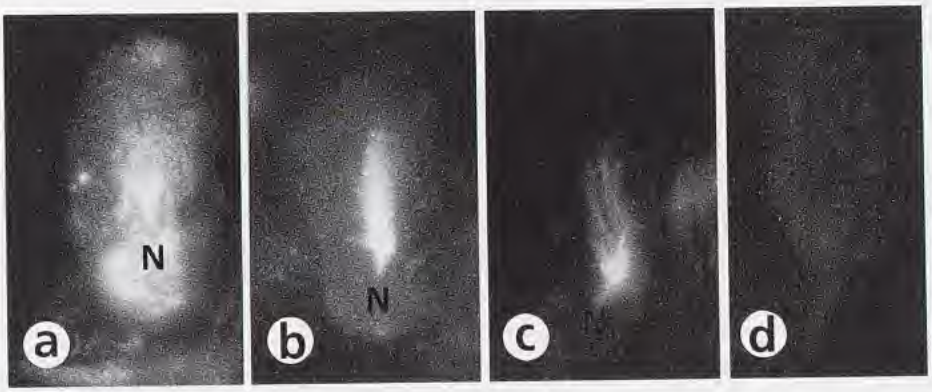


FIG. 2. — Immunocytochemistry of alpha-tubulin in spermatids of *Heligmosomoides polygyrus*. **a-d:** Successive stages of spermiogenesis, showing the decreased amount of tubulin in spermatids. The nuclear fossa is strongly labelled, thus indicating that the centriolar region located here may act as a microtubule organizing centre. Mature spermatozoa are not labelled and thus are not pictured here. a-d, x 2 500.

This labelling is consistent with the view that the centriolar region, located in the nuclear fossa, plays the role of a microtubule organizing centre (MTOC). However, centrioles in mature spermatids and spermatozoa are no longer labelled by the antibodies. This could be due to a penetration problem, since the centrioles are deeply located in the fossa in the mature spermatozoa and are embedded in an electron-dense material. An alteration of tubulin antigenicity in mature spermatozoa cannot, however, be excluded.

The labelling illustrated in Fig. 2 was performed with an anti-alpha tubulin antibody. The anti beta-tubulin antibody gives a similar pattern. The anti-acetylated tubulin antibody gave no labelling at any stage.

DISCUSSION

The spermatozoon of *Heligmosomoides polygyrus* has been previously described by WRIGHT & SOMMERVILLE [81, 84]. Our study confirms their observation on gross morphology and on the presence of microtubules in spermatids, which are no longer present in mature spermatozoa. However, the centrioles were not described in their study.

Centrioles have been described in nematode spermatozoa in a limited number of species (Table 2).

Source : MNHN, Paris

TABLE 2. — Centrioles in nematode spermatozoa.

Species	Centriole structure	Reference
Heterakis gallinorum	9 singlets	[40]
Dipetalonema viteae	9 singlets	[45]
Caenorhabditis elegans	9 singlets	[80]
Trichinella spiralis	9 singlets	[68]
Sphaerolaimus hirsutus	9 singlets	unpublished
Ascaris megalocephala	9 singlets	[22]
Nippostrongylus brasiliensis	9 singlets	[33, 83]
Gastromermis sp. (spermatid)	9 doublets	[53]
Heligmosomoides polygyrus	10 singlets	Present study

Table 2 demonstrates that singlets are the usual structure found in nematode spermatozoal centrioles. The description of doublets in *Gastromermis* refers only to spermatids [53] and the structure is not described in mature spermatozoa. It is not unlikely that the doublets are simplified into singlets in mature spermatozoa in this species; such a process is know in many Platyhelminthes (see [36]).

The number of singlets in nematode sperm centrioles is generally 9. The centriole of *Nippostrongylus* has been described as having 18 singlets by JAMUAR [33], but examination of the photographs suggests that it is made of 9 singlets separated by regular spaces, as later correctly described by WRIGHT & SOMMERVILLE [83]. The case of *Heligmosomoides polygyrus* described here is the first case with 10 singlets, and therefore 10-fold symmetry.

The 9-fold symmetry is almost ubiquitous in axonemes. A few exceptions have been noted: axonemes made up of 3 [54], 6 [62], 8 [55], 12 [5, 75], 13 [17, 86], 14 [5, 18], 16 doublets [18] have been described in spermatozoa of certain animals. Axonemes with a 9+"1" structure (nine doublets) but with centrioles made up of 18 singlets have been recently described in a flatworm [36]. The only known case of a ten-fold symmetry in a spermatozoal axoneme has been very recently described by DALLAI *et al.* [16] in the spermatozoa of a dipteran, which has a 10+0 structure. DALLAI *et al.* (1995) [16] have remarked that "in order to accommodate a tenth doublet, the axoneme either must increase its cross-sectional diameter or the doublets have to be more closely packed. The former alternative seems to be realized as the axoneme is seen to have an elliptic shape...". In *Heligmosomoides polygyrus* centrioles, both conditions (closer microtubules and elliptic shape) are found.

It is known that the ciliary apparatus is generally very reduced in nematodes [8]: flagella are absent from spermatozoa, motile cilia are unknown, and an axonemal structure is found only in sensory cells. Moreover, sensory cilia of nematodes often show a deviation from the 9+2 pattern. Cilia with 10+0 structure (10 peripheral doublets) have been described in larval *Haemonchus contortus* [60].

In spermatozoa which have a flagellum, the presence of a centriole is correlated with the existence of the flagellum. In the Nematoda, flagella are never present and the role of the centrioles may be questioned. Centrioles seem to act as MTOC during spermiogenesis, but might, on first consideration, appear redundant in mature sperm. Their presence in mature sperm may indicate a role during fertilization. However, the participation of the paternal centriole during fertilization is not ascertained in all animal species [61]. In the nematodes, a comparative study of the role of centrioles in fertilization in species with centrioles with 9 singlets and in *Heligmosomoides polygyrus* with 10 singlets would be interesting.

Source : MNHN, Paris

Spermatozoa of nematodes have been tentatively used for the understanding of phylogeny by BACCETTI *et al.* [6]. The position of the centrioles may provide additional characters. For instance, if we consider only spermatozoa with elongate shape, the position of centrioles is different in *Gastromermis*, where they are at the distal (posterior) tip of the nucleus [53], whereas they are at the anterior extremity of the nucleus in the Trichostrongyloidea [33, 44, 82-84]. The symmetry of the centrioles may provide further useful character.

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Source : MNHN, Paris

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Source : MNHN, Paris

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Source : MNHN, Paris

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Source : MNHN, Paris

Ultrastructure of Sperm and Sperm-Egg Interaction in Aculifera: Implications for Molluscan Phylogeny

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ABSTRACT

All Aculifera, other than neomenioids, fertilize externally with ectaquasperm but these vary greatly in structure between Polyplacophora and Aplacophora, reflecting different modes of fertilization. These structural differences provide us with an additional set of relatively conservative characters with which to examine the phylogeny of Aculifera in relation to other Mollusca and to Bilateria as a whole. Primitive chitons, perhaps similar to *Leptochiton asellus*, probably had ectaquasperm with a large acrosome that penetrated a smooth thick-hulled egg by the release of digestive enzymes and the extrusion of a perforatorium (as is typical of other molluscs). In the majority of extant chitons, such as *Cryptochiton stelleri*, the acrosome has been reduced to a tiny vesicle that digests a pore in the egg envelopes, through which a needle-like portion of the nucleus (replacing the perforatorium), penetrates to fuse with an egg microvillus. The egg hulls of these chitons are elaborated into complex spines or cupules, which may be open or closed. In closed cupule species sperm penetrate between cupules, whereas in open cupule species sperm are attracted inside the cupules to fertilize. Ectaquasperm of Chaetodermomorpha are highly unusual with many derived features indicating a long divergence of the group. Sperm exposed to egg water exhibited an elongation of the apical tube, which suggests a novel polymerization process since a typical subacrosomal granule is absent. Introsperm of Neomeniomorpha are similar to those of internally fertilizing prosobranch snails as well as some polychaete annelids, but are closest in morphology to the parasperm of *Protodrilus* sp., a primitive annelid. This provides additional support for the theory that molluscs and annelids are sister taxa and also suggests that internal, not external, fertilization, may be plesiomorphic to both groups. This would require secondary evolution of ectaquasperm, an idea that has also been suggested for teleost fishes to explain the occurrence of introsperm in basal groups. Furthermore, all extant Platyhelminthes have introsperm but only *Nemertoderma* sp., considered a basal member of the group, has a plausible acrosome, filiform nucleus and midpiece, and typical 9+2 flagellum. Platyhelminthes are widely regarded as basal to the Bilateria which suggests, in contradiction to the widely held belief that early bilaterians fertilized externally, that the common bilaterian ancestor of Platyhelminthes and Eubilateria fertilized internally with introsperm. This indicates that Bilateria are a monophyletic group, their basal members being united by the shared synapomorphies of similar introsperm and internal fertilization, which are absent in all extant Radiata.

RÉSUMÉ

Ultrastructure des spermatozoïdes et des interactions spermatozoïdes-oeuf chez les Aculifera: implications pour la phylogénie des Mollusques

Tous les Aculifera autres que les Neomenioidae ont une fécondation externe avec ectaquaspermatozoïdes dont la structure varie beaucoup entre les Polyplacophora et les Aplacophora, reflétant des modes différents de fécondation. Ces différences structurelles nous fournissent un ensemble supplémentaire de caractères relativement conservés avec lesquels on peut examiner la phylogénie des Aculifera en relation avec les autres Mollusques et l'ensemble des Bilateria. Les chitons primitifs, peut-être similaires à *Leptochiton asellus*, avaient probablement un ectaquaspermatozoïde avec un grand

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Source : MNHN, Paris

acrosome qui pénétrait un oeuf à coque lisse et épaisse grâce à la libération d'enzymes digestives et l'extrusion d'un perforatorium (situation typique des autres Mollusques). Dans la majorité des chitons, tels que *Cryptochiton stelleri*, l'acrosome a été réduit à une petite vésicule qui digère un pore dans les enveloppes de l'oeuf, à travers lequel une portion en aiguille du noyau (qui remplace le perforatorium) pénètre pour fusionner avec une microvillosité de l'oeuf. La coque de l'oeuf de ces chitons est compliquée par des épines ou des cupules, qui peuvent être ouvertes ou fermées. Dans les espèces à cupules fermées les spermatozoïdes pénètrent entre les cupules, alors que dans les espèces à cupules ouvertes les spermatozoïdes sont attirés à l'intérieur des cupules pour la fécondation. Les ectaquaspermatozoïdes des Chaetodermomorpha sont très différents, avec de nombreux caractères dérivés indiquant une longue divergence du groupe. Les spermatozoïdes exposés aux sécrétions des oeufs montrent une élongation du tube apical, qui suggère une polymérisation d'un nouveau type puisque le granule subacrosomien est absent. Les introspermatozoïdes des Neomeniomorpha sont similaires à ceux des Mollusques Prosobranches à fécondation interne et à certaines Annélides Polychètes, mais ont une morphologie très proche du paraspermatozoïde de *Protodrilus* sp., une Annélide primitive. Ceci fournit des arguments additionnels à la théorie selon laquelle les Mollusques et les Annélides sont des groupes-frères et suggère aussi que la fécondation interne, et non externe, pourrait être plésiomorphe dans ces deux groupes. Ceci demanderait une évolution secondaire de l'ectaquaspermatozoïde, une idée qui a déjà été suggérée chez les Poissons Téléostéens pour expliquer la présence d'introspermatozoïdes dans les groupes basaux. De plus, tous les Plathelminthes ont des introspermatozoïdes mais seul *Nemertoderma* sp., considéré comme un membre basal du groupe, a un acrosome plausible, un noyau et une pièce intermédiaire filiforme, et un flagelle typique 9+2. Les Plathelminthes sont généralement considérés comme étant à la base des Bilateria ce qui suggère, en contradiction avec la croyance largement répandue que les premiers Bilateria avaient une fécondation externe, que l'ancêtre commun des Plathelminthes et des Eubilateria avait une fécondation interne avec introspermatozoïde. Ceci indique que les Bilateria sont un groupe monophylétique, leurs membres basaux étant unis par des synapomorphies d'un introspermatozoïde similaire et de la fécondation interne, caractères qui sont absents chez tous les Radiata.

Aculifera consist of two classes, Polyplacophora (chitons) and Aplacophora (chaetoderms and neomenioids) [67, 69]. Among Metazoa, the most primitive groups, Porifera and Radiata, fertilize externally with sperm that have the typical shape, round head, four or five spherical mitochondria and a centriolar satellite complex that harnesses the flagellum at the base of the sperm. To avoid prior connotations of "primitive" and "modified" being associated with sperm shape, JAMIESON and ROUSE [47] devised a new system of nomenclature based on the mode of fertilization, in keeping with FRANZÉN's original hypothesis that sperm structure correlates with biology of fertilization [33, 34]. Two key terms emerged, "ectaquasperm" are characteristic of external fertilizers, whereas "introsperm" characterize internal fertilizers. This system provides for intermediate conditions such as "entaquasperm" [60, 61] and "ect-terrasperm" [80], as well as "plesiosperm" for the archetype [45].

Chitons and chaetoderms have external fertilization with ectaquasperm, although sperm structure and mode of fertilization vary greatly between the groups indicating a long divergence. Neomenioids fertilize internally with introsperm [32], which have a similar morphology to sperm of some primitive annelids [53], gastropods, such as Neritimorpha [15] and Caenogastropoda [13, 40], as well as other invertebrates [2, 3, 35]. The eggs of Aplacophora are relatively unspecialized in relation to surface features but in chitons the hull is usually elaborated into a series of complex spines or cupules, which may be closed or open to the external environment [21, 22, 29]. In hermaphroditic and brooding species the cupules tend to be reduced, sometimes to flattened plates [20, 29]. Self-fertilization is predominant in hermaphroditic species but sperm morphology is unchanged presumably because sperm encounter eggs in a sea water medium in the pallial grooves [20].

Aplacophorans are easily separated from the chitons on the basis of synapomorphies for distichous radula, reduced foot, gonads that open into the pericardium, and small posterior mantle cavity [67, 69]. Furthermore, neomenioids are quite distinct from chaetoderms on the basis of apomorphic characters for sperm structure, other aspects of reproductive biology, as well as general morphology [66, 67, 69]. The main problem arises in trying to distinguish between the different families of chitons.

Source : MNHN, Paris

Chiton phylogeny has been difficult to resolve because potentially useful characters such as radula morphology, which have an extensive fossil record, are virtually unchanged in the chitons [28, 31, 78]. Other characters such as girdle morphology and hull spine tip structure, are highly variable and may have arisen independently in several lineages, or may be polymorphic in a single taxon [28, 71]. Furthermore, previous classifications have been constructed as a series of progressive grades or ascending levels of organization rather than in a way to test phylogenetic affinities [28, 79].

Several authors advocate the use of a classification system for generic and higher taxa based on shell valve morphology alone, because these characters can be corroborated with the fossil record [for review, see 78]. However, as EERNISSE [28] pointed out, any phylogenetic analysis would be strengthened by inclusion of independent morphological characters to avoid coincidental resemblance of form (i.e. of shell valves). EERNISSE first revealed the potential advantages of using gill placement and morphology, as well as egg hull morphology, as independent character sets to test the validity of previous phylogenies based on shell valve morphology alone [28]. SIRENKO followed up on this and studied gill placement and egg hull morphology in 117 species of chitons many of them for the first time [71]. This analysis produced not only a re-arrangement of existing families but the addition of several new ones, including two new super-families, Tonicelloidea and Schizochitonoidea. Lepidochitoninae were included as a subfamily within Tonicellidae, in the superfamily Tonicelloidea. Furthermore, all chitons were grouped in three suborders, Lepidopleurina, Chitonina and Acanthochitonina.

This chapter examines sperm structure and sperm-egg interactions in Aculifera. It has been restricted largely to those species for which complete sperm and egg hull data are available. Thus, families for which sperm and egg data were not verifiable by the author have been omitted from the cladistic analysis. These include Ochmazochitonidae, Loricidae, Schizochitonidae, Juvenochitonidae, Schizoplacidae, and Cryptoplacidae (see [71]). In a few instances key species have been included from other studies to ensure a more complete analysis of a family. This analysis of new sperm and egg characters, when combined with existing morphological characters, has enabled a reassessment of phylogenetic relationships among Aculifera and between them and other taxa.

RESULTS

Sperm structure in Polyplacophora

Chiton sperm can be classified into one of four main types, depending on the presence of a typical acrosome, type of chromatin condensation, number and position of mitochondria, and presence of an axonemal fibrous complex. The appropriate families for each sperm type are listed on the first line, and follow the recent reclassification of chitons by SIRENKO [71], with the exception of Lepidochitonidae [see phylogeny].

Sperm type 1: (Acanthochitonidae, Mopaliidae, Tonicellidae, *Lepidochitona*). This sperm type is probably the most derived as well as the best characterized [16, 17, 19-22, 42, 64, 65]. A detailed description will be given based on this information and following the four arbitrary stages of spermiogenesis described elsewhere [19] and illustrated here in Figure 37. The other sperm types will then be compared to it.

At the onset of spermiogenesis tetrads of Stage A spermatids are interconnected by cytoplasmic bridges (Fig. 4). Chromatin in the spherical nucleus is patchy and granular in appearance. Two centrioles are positioned adjacent to the plasma membrane with the distal one being attached to it via the centriolar satellite complex and annulus (Fig. 1). The proximal centriole is perpendicular to the distal centriole and anterior to it. There are six or more

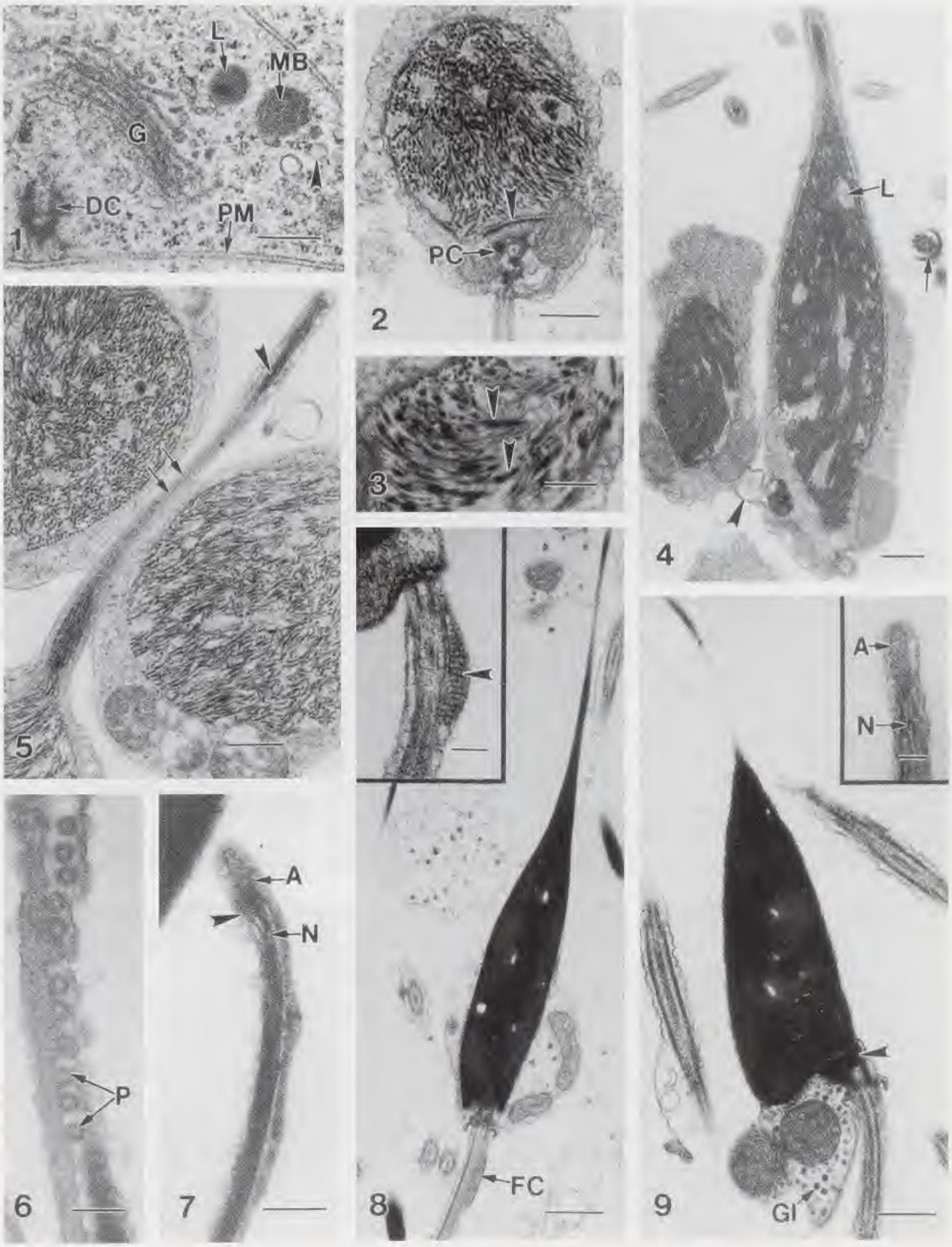
Source : MNHN, Paris

TABLE 1. — List of species used in the present study, classified according to SIRENKO [71].

Species studied	Family	Reference
Leptochiton asellus (Linnaeus, 1767)	Lepidopleuridae	[24, 42]
Callochiton castaneus (Wood, 1875)	Callochitonidae	[24, 42]
Chaetopleura apiculata (Say, 1834)	Chaetopleuridae	[19]
Acanthopleura granulata (Gmelin, 1791)	Chitonidae	[4, 24, 71]
Callistoplax crassicostatus Pilsbry, 1893	Callistoplacidae	[24, 28, 71]
Stenoplax conspicua (Pilsbry, 1892)	Ischnochitonidae	[24, 71]
Lepidozona retiporosa (Carpenter, 1864)	Ischnochitonidae	[24]
Chiton tuberculatus (Linnaeus, 1758)	Chitonidae	[24, 71]
Onithochiton quercinus (Gould, 1846)	Chitonidae	[24, 42, 71]
Ischnochiton albus (Linnaeus, 1767)	Ischnochitonidae	[24, 42, 71]
Lepidochitona dentiens (Gould, 1846)	Tonicellidae	[19, 28, 29]
Lepidochitona fernaldi Eernisse, 1986	Tonicellidae	[19, 20, 28, 29]
Tonicella lineata (Wood, 1815)	Tonicellidae	[19, 28]
Tonicella marmorea (Fabricius, 1780)	Tonicellidae	[19, 71]
Nuttalina flexa (Carpenter, 1864)	Mopaliidae	[24, 28, 71]
Mopalia muscosa (Gould, 1846)	Mopaliidae	[19, 21]
Mopalia lignosa (Gould, 1846)	Mopaliidae	[21, 28]
Mopalia ciliata (Sowerby)	Mopaliidae	[21]
Katharina tunicata (Wood, 1815)	Mopaliidae	[19, 28, 71]
Acanthochitona viridis (Pease, 1872)	Acanthochitonidae	[19]
Acanthochitona pygmaea (Pilsbry, 1893)	Acanthochitonidae	[24]
Cryptochiton stelleri (Middendorff, 1857)	Acanthochitonidae	[19, 28, 71]
Chaetoderma argenteum Heath, 1911	Chaetodermomorpha	[18]
Chaetoderma canadense Nierstrasz, 1902	Chaetodermomorpha	[24]
Epimenia australis (Thiele, 1897)	Neomeniomorpha	[22, 23]

FIGS 1-9. — Transmission electron micrographs of spermiogenesis in Polyplacophora. **1:** Stage A type 1 spermatid of *Tonicella marmorea* showing Golgi body (G) and associated lysosome (L), multivesicular body (MB) and vesicles (arrowhead). Distal centriole (DC) is attached to plasma membrane (PM) by the annulus. Scale bar = 0.2 µm. **2:** Longitudinal section (L.S.) of stage B type 2 spermatid of *Lepidozona retiporosa* showing condensation of chromatin in 20 nm fine fibres. The proximal centriole (PC) is attached by fibrogranular secretions to the thickened region of nuclear envelope (arrowhead). Scale bar = 1 µm. **3:** L.S. stage C type 2 spermatid of *L. retiporosa*, showing formation of 70 nm coarse fibres. Scale bar = 0.5 µm. **4:** L.S. stage C type 1 spermatid of *Cryptochiton stelleri* showing tight spiralling of fine chromatin fibres. Coarse fibre stage is absent. Note: cytoplasmic bridge joining spermatids (arrowhead); lacuna (L) in nucleus and cross section of flagellum at level of annulus (small arrow). Scale bar = 0.2 µm. **5:** L.S. stage C spermatid of *Tonicella lineata* with fine chromatin fibres twisted anteriolaterally forming extension of nucleus in anterior filament (arrowhead). Two microtubules of manchette surrounding nucleus are visible in superficial section (arrows). Scale bar = 0.5 µm. **6:** Stage D spermatid of *C. stelleri* showing aggregation of nucleopores (P) where nucleus extends into anterior filament. Scale bar = 0.3 µm. **7:** Tip of anterior filament of stage D type 1 spermatid of *Mopalia muscosa* showing acrosome (A) separated from nuclear extension (N) by basal (= subacrosomal) plate (arrowhead). Scale bar = 0.3 µm. **8:** L.S. stage D type 1 spermatid of *M. muscosa* in longitudinal section showing basic form of sperm and position of fibrous complex (FC). Scale bar = 0.2 µm. **Inset**: Close-up of fibrous complex (arrowhead). Scale bar = 0.2 µm. **9:** L.S. stage D type 2 spermatid of *Chaetopleura apiculata* in longitudinal section showing basic form of sperm with large round posterior mitochondria, surrounded by glycogen granules (Gl) on opposite side to basal body (arrowhead). Scale bar = 0.5 µm. Inset: Tip of anterior filament of *L. retiporosa* showing acrosome (A) atop nuclear extension (N). Scale bar = 0.1 µm.

Source : MNHN, Paris



Source : MNHN, Paris

mitochondria at this stage but as spermiogenesis proceeds they migrate posteriorly and fuse to form between 4 and 7 in the mature sperm. A prominent Golgi body secretes several small proacrosomal vesicles and is usually associated with a lysosome and multivesicular body (Fig. 1). Stage B in all chitons is characterized by the development of the centriolar fossa at the base of the nucleus accompanied by a thickening of the outer nuclear membrane (Fig. 2). The centrioles migrate into this region and the proximal centriole becomes attached by fine fibrous strands to the thickened nuclear membrane (similar to Fig. 2). The flagellum elongates posteriorly. Chromatin condensation progresses forming fine fibres about 20 nm in diameter, which later in this stage become twisted anteriolaterally towards the pointed end of the nucleus (Fig. 5). A microtubular manchette emanates from the centrioles, twists around the nucleus and extends into the pointed tip of the spermatid (Fig. 5). In the Stage C spermatid two granules begin to develop in the acrosome by the fusion of proacrosomal vesicles. The nuclear filament becomes more elongate (Fig. 5) and the nucleopores aggregate at its base (Fig. 6). The rest of the nucleus appears to be devoid of nucleopores at this stage. Glycogen granules are formed in the midpiece, near the Golgi body and mitochondria. The basal body is slightly offset and mitochondria begin to group above it on the same side, and below it on the opposite side. In Stage D the spermatids mature and the microtubular manchette disappears. The acrosome, containing two granules, is separated from the nuclear filament by a basal (= subacrosomal) plate (Fig. 7). The nucleus and nuclear filament become homogeneously dense with occasional lacunae in the main body (Fig 8). The nucleus is bullet-shaped and subtends an offset basal body comprising proximal and distal centrioles interconnected by fibrogranular secretions. Mitochondria are found laterally above the centrioles and basally opposite them. The centriolar satellite complex and annulus are housed in the collar, which is an evagination of plasma membrane that extends posterior to the distal centriole and around the axoneme. A fibrous complex forms adjacent to the axoneme near the annulus, on the same side as the basal body (Fig. 8, inset). The elongate flagellum terminates in a narrow endpiece, which contains only the central microtubules and is similar in length to the nuclear filament. The Golgi body, rough endoplasmic reticulum, lysosome, multivesicular body, as well as excess cytoplasm are eliminated via the cytoplasmic bridge into a central cytoplasmic droplet, which is sloughed off thus freeing the four mature spermatozoa of the cohort.

Sperm type 2: (Chaetopleuridae, Chitonidae, Callistoplacidae, Ischnochitonidae). Type 2 chiton sperm has been described previously for 12 species [4, 19, 42, 63-65], and is represented here by *Lepidozona retiporosa*. Type 2 sperm develop similarly to Type 1 with the following exceptions: firstly, following the fine fibre phase (fibres are 20 nm in diameter) of chromatin condensation (Fig. 2), there is a coarse fibre phase (fibres are 70 nm in diameter) (Fig. 3), before the nucleus becomes homogeneously dense (Fig. 9). Secondly, the midpiece has a distinctive

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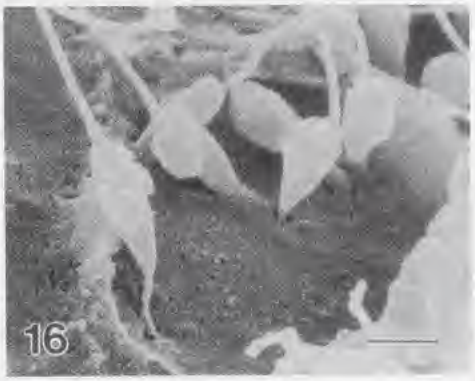
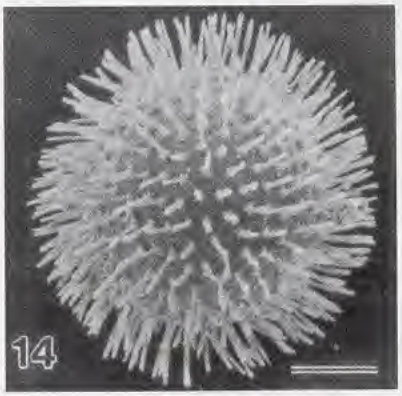
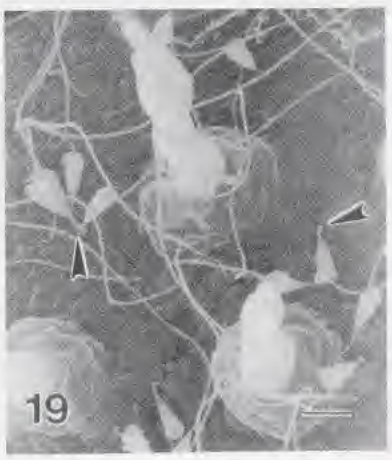
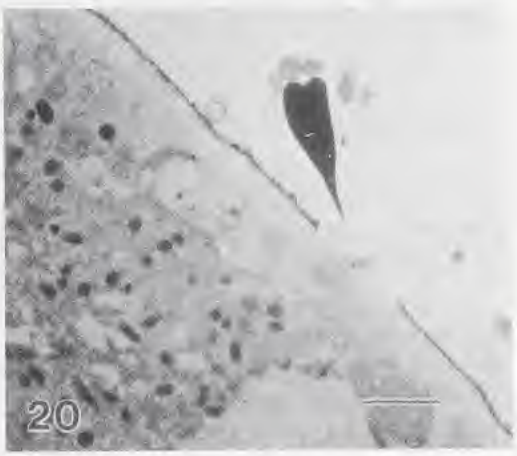
FIGS 10-20. — Sperm-egg interactions in Polyplacophora. **10:** Mature open cupule egg of *Mopalia ciliata*. Scale bar = 50 µm. **11:** Open cupules of polyspermic egg of *M. muscosa*. Scale bar = 20 µm. **12:** Polyspermic egg of *M. muscosa* with cupules removed revealing preponderance of sperm inside cupule profiles. A few sperm penetrate area between cupules. Scale bar = 10 µm. **13:** Mature closed cupule egg of *Lepidochitona dentiens*. Scale bar = 50 µm. **14:** Mature spinous egg of *Stenoplax conspicua*. Scale bar = 50 µm. **15:** Polyspermic eggs of *S. conspicua* with sperm between spines. Bifurcations of spines now are closed, but were spread apart when spawned. Scale bar = 10 µm. **16:** Polyspermic egg of *M. muscosa*: sperm penetrating hull inside cupule. Scale bar = 2 µm. **17:** Light micrograph of 1 µm section of sperm of *T. lineata* penetrating hull and vitelline layer to inject nuclear material into egg (arrowhead). Scale bar = 2 µm. (From [16]). **18:** Portion of maturing egg of *Acanthopleura granulata*, showing polymorphic spines. Short bifurcating spines occurring between medium to long single tipped spines with caps. Scale bar = 10 µm. **19:** Polyspermic egg of *S. conspicua* in plan view showing sperm penetrating hull at junctions of cupule bases (arrowheads). Scale bar = 5 µm. **20:** T.E.M. of fertilizing sperm penetrating hull of *S. conspicua*. Characteristic dense outer layer of hull, has been dissolved around penetrating sperm. Scale bar = 2 µm.

Source : MNHN, Paris









Source : MNHN, Paris

appearance because of the lack of lateral mitochondria and the position of basal mitochondria (Fig. 9). Frequently two mitochondria are adjacent to the nucleus with a third one below them. This extends the midpiece and collar posteriorly. Thirdly, the basal body is more offset than in Type 1. This and the position of mitochondria gives the Type 2 sperm its characteristic shape (Fig. 9). Fourthly, the fibrous complex is absent or poorly developed, but at least in some species the plasma membrane is thickened at the same site as the fibrous complex of Type 1 sperm. The acrosome is more uniform in appearance, lacking distinct apical and basal granules (Fig. 9, inset).

Sperm type 3: (Callochitonidae). The third type of sperm has been described only in Callochitonidae [42, 64], represented here by *Callochiton castaneus*. Sperm of Hanleyidae, a fairly primitive family, have not yet been examined.

Type 3 sperm is characterized by the plesiomorphic condition of a central basal body and flagellum, surrounded by five mitochondria at the base of the nucleus. The mitochondria are slightly derived because two of them are elongate whereas three are spherical [42].There is no fibrous body or unilateral reinforcement of the plasma membrane near the annulus. In other respects the sperm is similar to Type 2 with a similar pattern of chromatin condensation.

Sperm type 4: (Lepidopleuridae). This sperm type, considered plesiomorphic to chitons, is known only from the species, *Leptochiton asellus* [42]. The sperm is unique among chitons in having a fairly typical molluscan acrosome. A detailed diagram summarizing spermiogenesis in *L. asellus* is given in Figure 36 (based on [42] and unpublished observations).

In Stage A spermatids a large proacrosomal vesicle (1 μm in diameter) is evident posterior to the nucleus and connected to the plasma membrane by flocculent material, as occurs in aplacophorans and other molluscs. During Stages B and C the nucleus becomes oval and chromatin condensation proceeds from granular to short fine fibres 20 nm in diameter, then to short coarse fibres, 70 nm in diameter. The fibres appear much shorter than in other chiton groups, reflecting variation in the pattern of condensation. The mitochondria fuse to form five spheres surrounding a central basal body and unspecialized flagellum. In Stage C the proacrosomal vesicle, independent of the Golgi body, differentiates into the elongate acrosome cone and subacrosomal granule, which becomes separated from the nucleus by the subacrosomal plate. The subacrosomal granule apparently lacks filaments, as in some gastropods. In Stage D, the nucleus becomes homogeneously dense and bullet-shaped with no anterior filament. The flagellum terminates in a short filamentous endpiece containing only the central microtubules.

Sperm-egg interaction in Polyplacophora

Chiton eggs are surrounded by a vitelline layer as well as a hull, both of which appear to be secreted by the egg [58, 59]. With few exceptions the hulls of chiton eggs are characterized by elaborate projections which vary from spinous to cupular [16, 17, 20-22, 28, 29, 31, 43, 56, 58, 70, 71]. By contrast the hull of *Leptochiton asellus* (Lepidopleuridae) is smooth [42]. In brooding forms, which include several hermaphroditic species, the cupules are often reduced but retain the basic morphology of the group [20, 28, 29]. There are three main types of sperm-egg interaction which correlate with egg hull structure as follows: Type 1. Cupular projections; Type 2. Spinous projections; Type 3. Smooth hull. Variation in the size, shape and structure of hull projections has profound influences on; the site and mechanism of fertilization [16, 17, 20-22], the drag on the egg and therefore its sinking rate [21], the aggregation of eggs in strings and clusters and attachment to substrates [28, 29, review: 56] and therefore the target size for sperm, as well as the limitation of numbers of eggs that can be brooded [20, 28, 29].

Egg type 1: Cupular projections. (Lepidochitonidae, Tonicellidae, Mopaliidae, Acanthochitonidae). In cupular species the projections may be open or closed, which directly influences where the sperm penetrate the egg. In species with open cupules, such as in *Mopalia ciliata*, (Fig. 10), sperm preferentially swim inside the cupules, become segregated into one of seven channels and penetrate the hull where it overlies the egg membrane (Figs 12, 16). At this

Source : MNHN, Paris

point the hull is thinnest. The hull can be penetrated between cupules but this is rare and has only been observed in polyspermic eggs, created artificially by using a sperm concentrate (Fig. 12). No micropores have been observed in the hull of these species. The acrosome appears to contain two granules, which in *Tonicella lineata* are exhausted sequentially as the sperm penetrates the two egg envelopes, fuses with an egg microvillus and injects the nuclear material into the egg [16, 17] (Fig. 17).

During ontogeny cupules are covered by follicle cells [58, 70], which are thought to be involved in their morphogenesis but not their secretion [58, 59] distinguishing this envelope from the chorion of other animal groups. An immature open cupule egg can easily be mistaken for a closed cupule one. A good example is *Tonicella lineata*, which only becomes obviously open cupule in form when it sheds follicle cells close to spawning [16, 17]. Thus it is very important to make definitive observations only on naturally spawned eggs. Chitons collected during the breeding season [review: 56] may be kept in culture dishes in running sea water, until spawning occurs. Usually this happens one or two days after collection. Males usually spawn first and appear to stimulate females [56]. In species with closed hull cupules, such as *Lepidochitona dentiens* (Fig. 13), sperm must penetrate the egg envelopes between projections. In *L. dentiens*, micropores are present between cupules in the mature egg that provide sperm with direct access to the vitelline layer [20, 21]. Preliminary evidence suggests that sperm acrosomes may be further simplified in this and similar species by having a single granule [21]. In the brooder *L. fernaldi*, the structure of the egg is the same as *L. dentiens* except that cupules are reduced to flattened plates [20].

Egg type 2: Spinous Projections. (Hanleyidae, Callochitonidae, Schizochitonidae and Loricidae [71]; Chaetopleuridae, Chitonidae, Ischnochitonidae, Callistoplacidae). In chitons with spinous projections, such as *Stenoplax conspicua* (Fig. 14), the sperm penetrates the hull between spines, frequently at the junction of their bases (Figs 15, 19). Spines assume a wide variety of forms from complex, as in *Chaetopleura apiculata*, to simple as in *Hanleyella asiatica* [71]. Simple spines have a wide variety of tip formations, including one to seven-fold symmetry. Spinous tips may be pointed and bifurcate, as in *S. conspicua* (Fig. 15) or petalloid, as in *Chiton tuberculatus*. However, there appears to be considerable polymorphism within genera [71], making it difficult to use tip form as a character in phylogenetic analysis. The egg hull of *S. conspicua* (and possibly other spinous hulled species), has an outer dense layer in addition to the typical hull. This thin dense layer is digested by the entrance of the sperm, presumably because certain acrosomal enzymes are released (Fig. 20). The area of the hull disrupted by sperm entrance is much greater than the width of the anterior filament and cannot be accounted for just by gaps between spine bases, visible in some micrographs (Fig. 19).

Egg type 3: Smooth Hull. (Lepidopleuridae). In *Leptochiton asellus*, the only chiton species known with a smooth-hulled egg, fertilization is presumed to occur anywhere on the egg surface. However, sperm-egg interaction has not been observed in any lepidopleurids. The presence of a typical molluscan acrosome is coincident with an egg hull that is ten times thicker than in other chitons and probably reflects penetration by a perforatorium as is typical of other molluscs and invertebrates [42]. For these and other morphological reasons (see phylogeny section), Lepidopleuridae are considered basal to Polyplacophora.

Sperm structure and sperm-egg interaction in Aplacophora

Class Aplacophora may be divided into two subclasses, the Neomeniomorpha and the Chaetodermomorpha [67, 69]. Neomenioids are considered basal to Aculifera and to Mollusca as a whole, based on recent morphological [67] as well as molecular analyses [62], whereas chaetoderms are considered to be derived, having diverged early from the molluscan lineage [69].

Neomenioids and chaetoderms have radically different modes of reproduction. Neomenioids are monoecious and fertilize internally, with introsperm being stored in seminal

Source : MNHN, Paris

receptacles [68, 69]. Chaetoderms are dioecious and fertilize externally, by releasing ectaquasperm into the ambient sea water [18, 69]. These sperm types are quite different from those of chitons.

Sperm of Neomeniomorpha: Epimenia australis. Spermiogenesis in *E. australis* is summarized diagrammatically in Fig. 38. Spermiogenesis of the introsperm of *Epimenia* is very similar to that of the parasperm of the primitive annelid, *Protodrilus*, as well as introsperm of some prosobranchs [13, 15, 40]. Stage A is similar to what has been described for chitons. In stage B the sperm still has the plesiosperm form (Fig. 21). During this stage the centrioles migrate to the centriolar fossa dragging the plasma membrane with them and causing it to invaginate where it is attached to the annulus. This creates a temporary flagellar canal. The mitochondria move posteriorly and begin to fuse at the base of the nucleus, initially into four or more spheres. At this time a Golgi body is posterior to the nucleus and mitochondria (Fig. 21). In stage C, chromatin condensation progresses from granular to fine fibres 20 nm in diameter, which become twisted as the nucleus elongates (Fig. 22). During this stage the Golgi body secretes numerous vesicles which contribute to a large mass of dense granular material at the base of the nucleus around the axoneme. The annulus breaks away from the distal centriole and begins migrating posteriorly towards the junction between midpiece and glycogen-piece. The dense granular mass becomes reorganized around the proximal and distal centrioles where it contributes to the formation of the basal body as well as to the adjacent peribasal body. Similar granular secretions are organized into a spiral ridge around the axoneme down to the annulus (Fig. 38C).

The Golgi body plays an active role in forming the spiral ridge much like that observed previously in *Nerita picea* [15]. Subsequently, the Golgi body migrates again to the apex of the sperm adjacent to the acrosome cone, which to this point has been developing in the absence of the Golgi body (Fig. 38C). An extracellular striated cone, coated with fine fibrous strands, begins

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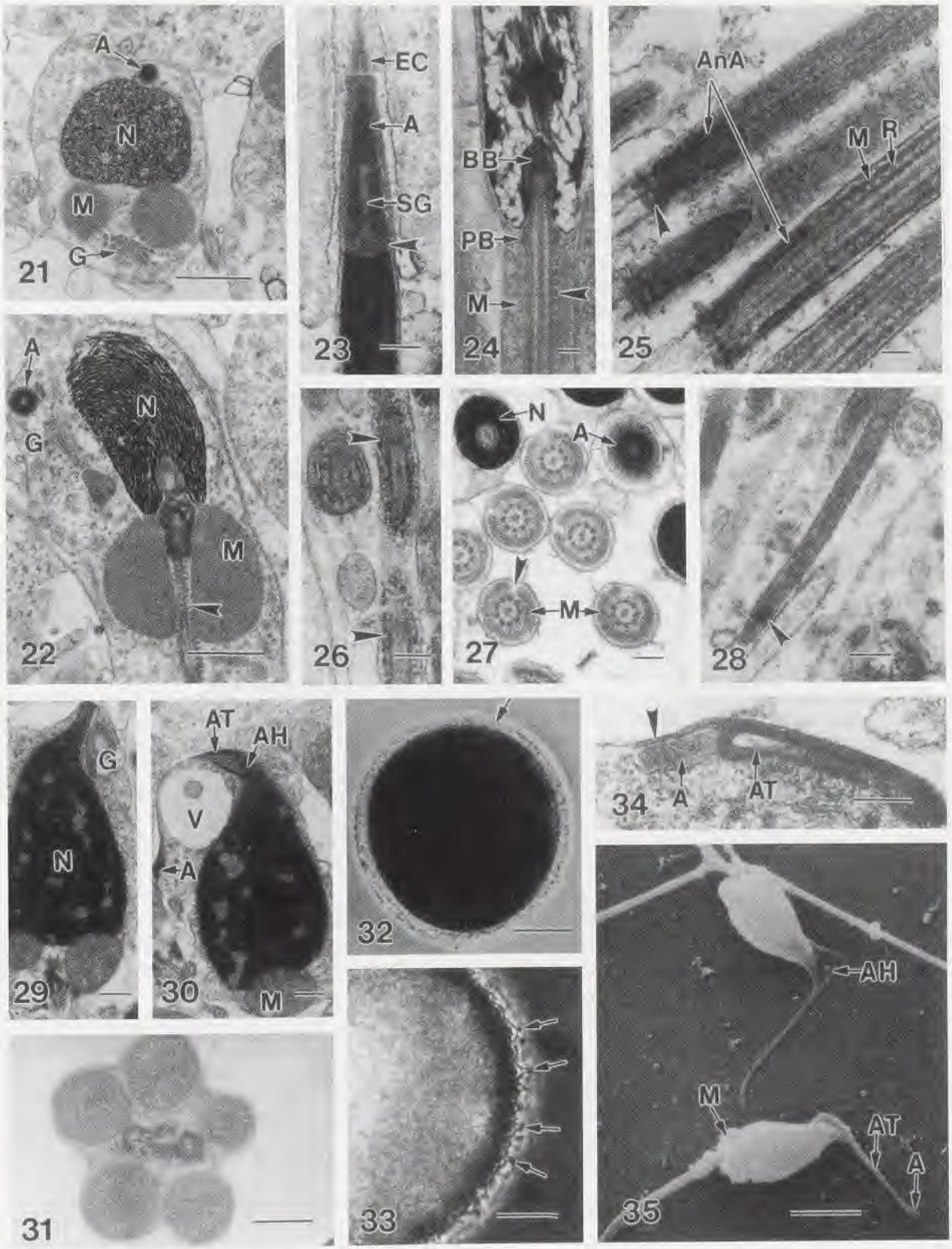
FIGS 21-28. — Spermiogenesis in Neomeniomorpha: *Epimenia australis*. **21:** L.S. stage B spermatid showing basic plesiosperm form. Apical acrosome (A), Golgi body (G) and spherical mitochondria (M) at base of nucleus containing granular chromatin (N). Scale bar = 1 µm. **22:** L.S. stage C spermatid showing twisting and spiralling of chromatin fibres in nucleus (N). Acrosome (A) is at tip of spiralled nucleus (out of section). Golgi body (G) is once again associated with the acrosome (or else there 2). Large fused mitochondria (M) are beginning to elongate around axoneme, which has been reinforced by spiral ridge (arrowhead). Scale bar = 1 µm. **23:** Stage D spermatid. L.S. of acrosome (A), subtending sub-acrosomal granule (SG) and separated from dense nucleus by sub-acrosomal plate (arrowhead). Note: striated extracellular cone (EC) on thickened membrane at tip of acrosome. Scale bar = 0.2 µm. **24:** L.S. stage D spermatid showing basal body (BB) in posterior indentation of nucleus, which is blocked by peri-basal body (PB). Note also: mitochondria (M) and sections of spiral ridge (R). Scale bar = 0.2 µm. **25:** Stage D spermatid. L.S. of junctions between mid-piece and glycogen piece showing annulus adjuncts (AnA), mitochondrion (M), spiral ridge (R) and annulus (arrowhead). Scale bar = 0.2 µm. **26:** Stage D spermatid. L.S. glycogen piece showing glycogen granules (arrowheads). Scale bar = 0.3 µm. **27:** Cross Section (C.S.) stage D spermatids at level of acrosome (A), nucleus (N) and mitochondrion (M). Note single break in mitochondrion (arrowhead), which forms longitudinal slit. Scale bar = 0.2 µm. **28:** L.S. end-piece of stage D spermatids, showing density (arrowhead) at termination of central microtubules. Scale bar = 0.2 µm.

FIGS 29-35. — Sperm and eggs of Chaetodermomorpha. **29:** Stage C spermatid of *Chaetoderma canadense* with granular chromatin in nucleus (N), Golgi body (G) is in process of forming apical horn and apical tube in anterior of spermatid. Scale bar = 0.5 µm. **30:** Stage C spermatid of *C. canadense* showing acrosome (A) at tip of apical tube (AT), that caps apical horn (AH). Apical tube is projected over large Golgi-derived vesicle (V). One of five mitochondria (M) lies at base of nucleus. Scale bar = 0.5 µm. **31:** C.S. mid-piece of *C. argenteum* showing five mitochondria encircling centrioles. Scale bar = 0.5 µm. **32:** Light micrograph of mature egg of *C. argenteum* with apparent hull (arrow). Scale bar = 30 µm. **33:** Enlarged area of *C. argenteum* egg showing periodic bumps with short spinous or tubular projections (arrows). Scale bar = 20 µm. **34:** L.S. of acrosome (A) and apical tube (AT) of *C. canadense*. Note attachment of acrosome to plasma membrane (arrowhead). Scale bar = 0.2 µm. **35:** S.E.M. of *C. argenteum* sperm exposed to egg water for ten minutes. Apical tube (AT) of upper sperm is longer than lower sperm. Elongation can be at least 1.5x original length. Note also apical horn (AH), acrosome (A) and mitochondria (M). Scale bar = 2 µm.

Source : MNHN, Paris



Source : MNHN, Paris

to form near the tip of the acrosome, between the sperm and the somatic (Sertoli) cell. Its function is unknown.

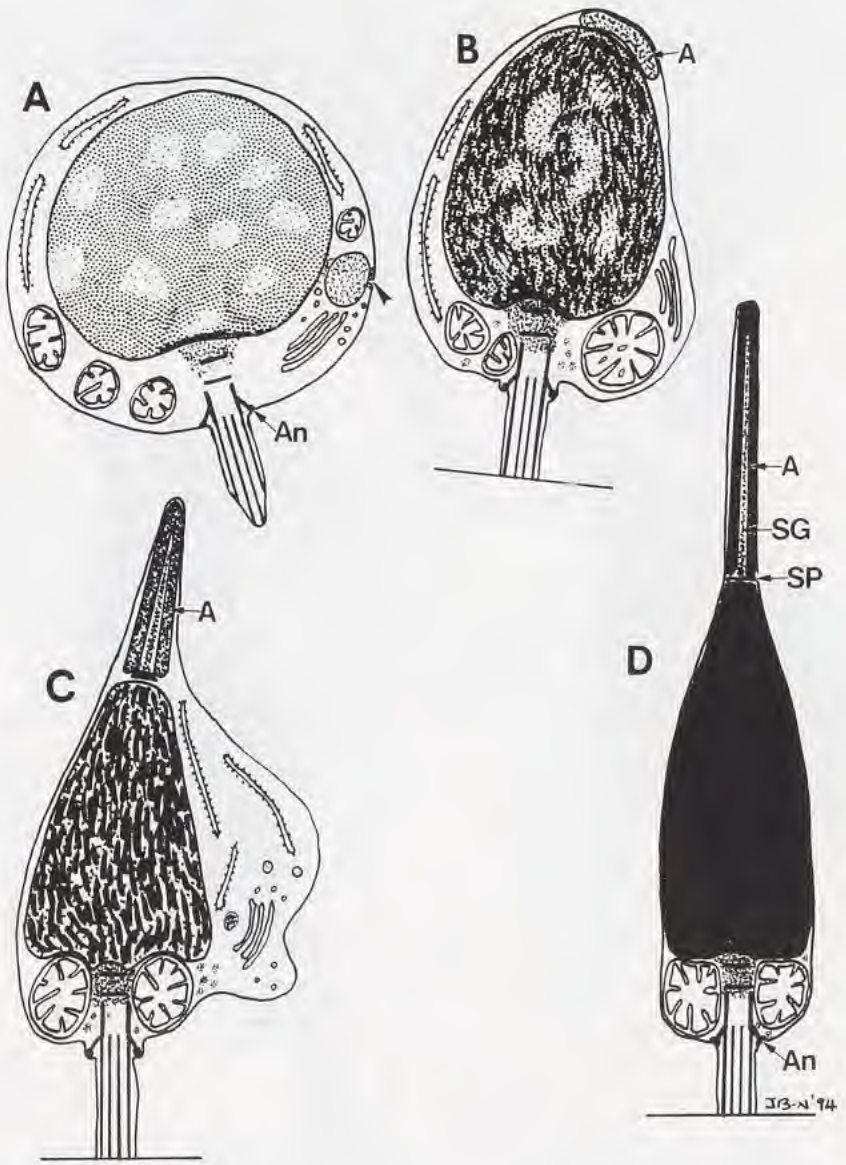
The basal body becomes housed in a posterior indentation of the nucleus, which is closed off by the peribasal body (Fig. 24). The annulus breaks away from its connections to the distal centriole and leads the posterior migration of the mitochondria. The acrosome cone is fully developed and subtends a subacrosomal granule, separated from the dense nucleus by a subacrosomal plate. The two mitochondria begin to elongate and later fuse to form a single Nebenkern enclosing the axoneme but for one longitudinal slit down its length (Fig. 27). In stage D the mitochondrion extends from the base of the nucleus to the tip of the annulus adjuncts, which are two hollow, semicylindrical structures that form above the annulus on either side of the axoneme (Fig. 25). The annulus occupies an invagination of the plasma membrane at the junction between midpiece and glycogen piece (a site typical of many metazoan introsperm) (Fig. 25). The glycogen piece is filled with granules of glycogen (Fig. 26), which tend to be grouped over the nine axonemal doublet microtubules. This aggregation is similar but less ordered than in gastropods. The axoneme tapers towards the endpiece, where it is reduced to just the central microtubules that terminate in a characteristic density (Fig. 28).

Sperm-egg interaction in Neomeniomorpha: Sperm-egg interaction in *Epimenia* has not been observed. The outer egg envelope appears to be unspecialized, which suggests that fertilization may occur anywhere on its surface. Fertilized eggs are brooded prior to release as juveniles [68].

Sperm of Chaetodermomorpha: Chaetoderma canadense, *and* C. argenteum. See diagrammatic summary of spermiogenesis in Fig. 39. The ectaquasperm of *C. argenteum* [18] and *C. canadense* [22] are highly unusual. Spermiogenesis begins in a similar way to *Leptochiton* but after forming the proacrosomal vesicle the Golgi body migrates to the apex of the nucleus (Fig. 29) where it becomes involved in producing three key structures: the apical horn, the apical (dense) tube and a large vesicle below the tip of the nucleus (Fig. 30). The apical tube grows out over the large vesicle to contact the proacrosome on the plasma membrane (Fig. 30). Glycogen granules are deposited around the apical horn as well as the mitochondria. At this time there are two flagella, proximal and distal, produced by the respective centrioles. The distal flagellum encircles the nucleus and exits posteriorly. The proximal flagellum grows anteriorly through the cytoplasm and exits at the tip of the nucleus, alongside the developing apical tube, perhaps acting as a guide [18]. Mitochondria fuse to form five spheres surrounding the centrioles at the base of the nucleus. At the completion of spermiogenesis the proximal flagellum separates at the centriole and is eliminated anteriorly with the residual cytoplasm (including Golgi body, large vesicle, lysosome, multivesicular body, endoplasmic reticulum, etc.) [18]. Conversely, in most Metazoa the residual cytoplasm is eliminated from the midpiece region. The distal flagellum unwinds and exits posteriorly in the usual position for molluscs. The chromatin condenses from granular, through patchy, to homogeneously dense with a few lacunae in the mature spermatid [18]. The distal centriole is harnessed to the plasma membrane by the annulus via the centriolar satellite complex, which typifies ectaquasperm [74] and is plesiomorphic for introsperm [2, 73, 77]. The axoneme has a typical 9+2 configuration and terminates in just the two central microtubules as in other Aculifera.

The mature sperm is easily mistaken for a chiton sperm because the apical tube resembles the anterior filament (Fig. 35) [32]. However, the apical extension is directed anteriolaterally and is of completely different construction [18]. It contains no nuclear material and comprises two unique structures, the apical horn and apical tube (Figs 30, 35), the functions of which remain unknown. There is, however, little doubt that the acrosome lies at the tip of the apical tube, as this vesicle forms from the Golgi body in much the same way as proacrosomes of some other molluscs, including *Leptochiton*.

Source : MNHN, Paris



FIGS 36-39. Diagrammatic representation of four arbitrary stages (A-D) of spermiogenesis in Aculifera following descriptions given in text.

FIG. 36. — Spermiogenesis in *Leptochiton asellus*. **A**: Pro-acrosome (arrowhead) formed from Golgi vesicles, is migrating to apex of nucleus along plasma membrane. Annulus (An) connects distal centriole to plasma membrane. Nucleus contains granular chromatin and in the cytoplasm are several small mitochondria and rough E. R. **B**: Pro-acrosome (A) is atop nucleus, chromatin is forming short fine fibres in nucleus. Mitochondria are at base of nucleus with Golgi body. **C**: Acrosome (A) is elongating in absence of Golgi body. Chromatin fibres are thicker and fused, enlarged mitochondria surround centrioles. **D**: Fully developed acrosome subtends a subacrosomal granule and is separated from dense nucleus by sub-acrosomal plate. Five uniform spherical mitochondria surround centrioles. Annulus (An) attaches distal centriole to plasma membrane via satellite complex [24, 42].

Source : MNHN, Paris

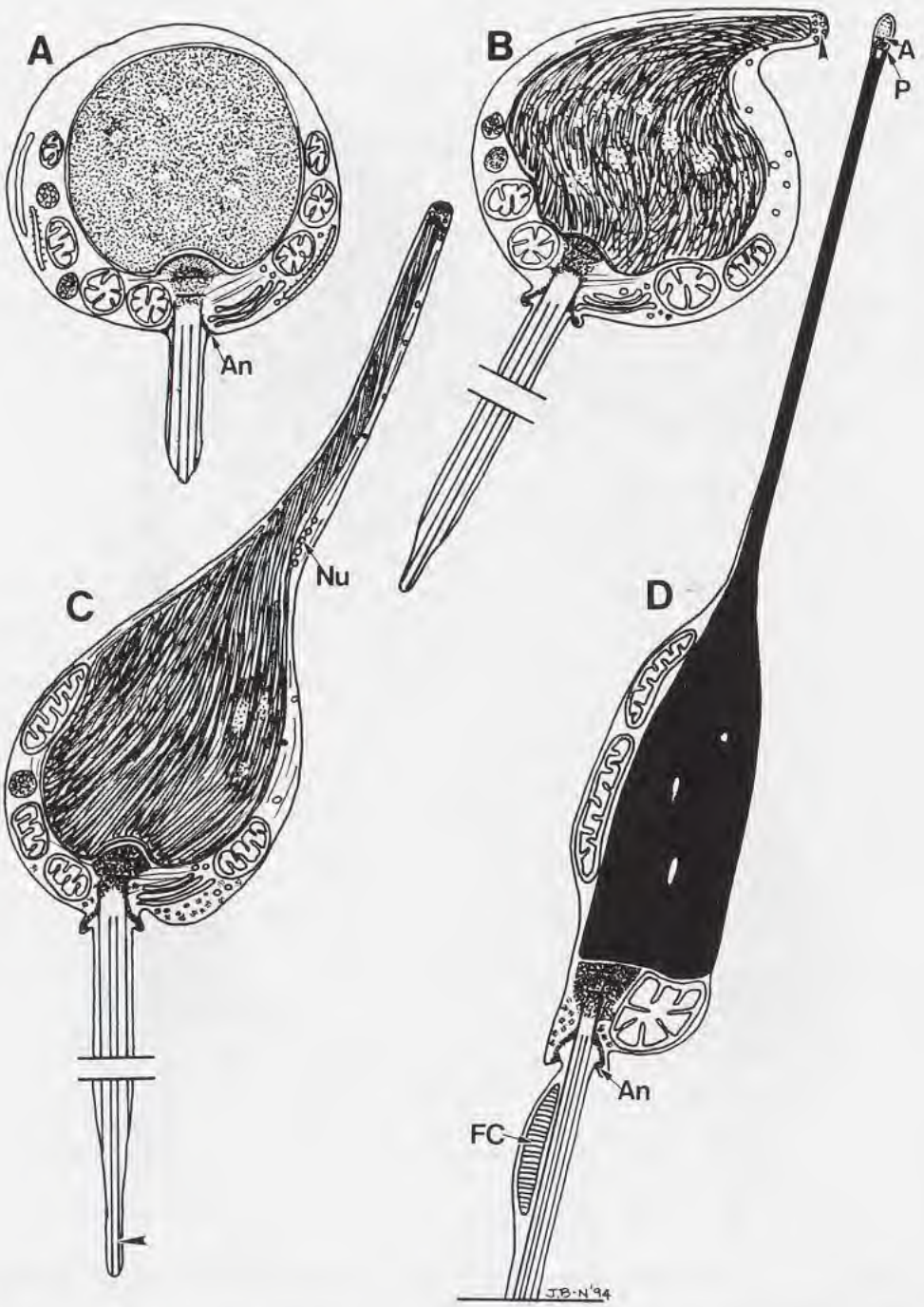


FIG. 37. — Spermiogenesis in *Cryptochiton stelleri*. **A**: Similar to *L. aseilus* but no pro-acrosome forms. Individual pro-acrosomal vesicles are visible near Golgi body. **B**: Chromatin condensing in fine fibres is twisting anterio-laterally in nucleus. Proacrosomal vesicles are visible at apex of spermatid (arrowhead). Mitochondria have accumulated at base of nucleus with Golgi body. **C**: Anterior filament is elongating with acrosome forming at its tip. Nucleopores (Nu) have aggregated at base of anterior filament. Tip of tail contains only central microtubules (arrowheads). **D**: Bullet shaped sperm has long needle-like filament with acrosome (A) at its tip. Acrosome is separated from dense nuclear extension by a basal (= subacrosomal) plate (P). Mitochondria are positioned laterally and below nucleus, adjacent to centrioles. Annulus (An) attaches distal centriole to plasma membrane. Fibrous complex (FC), characterizing Type 1 sperm, is visible below annulus.

Source : MNHN, Paris

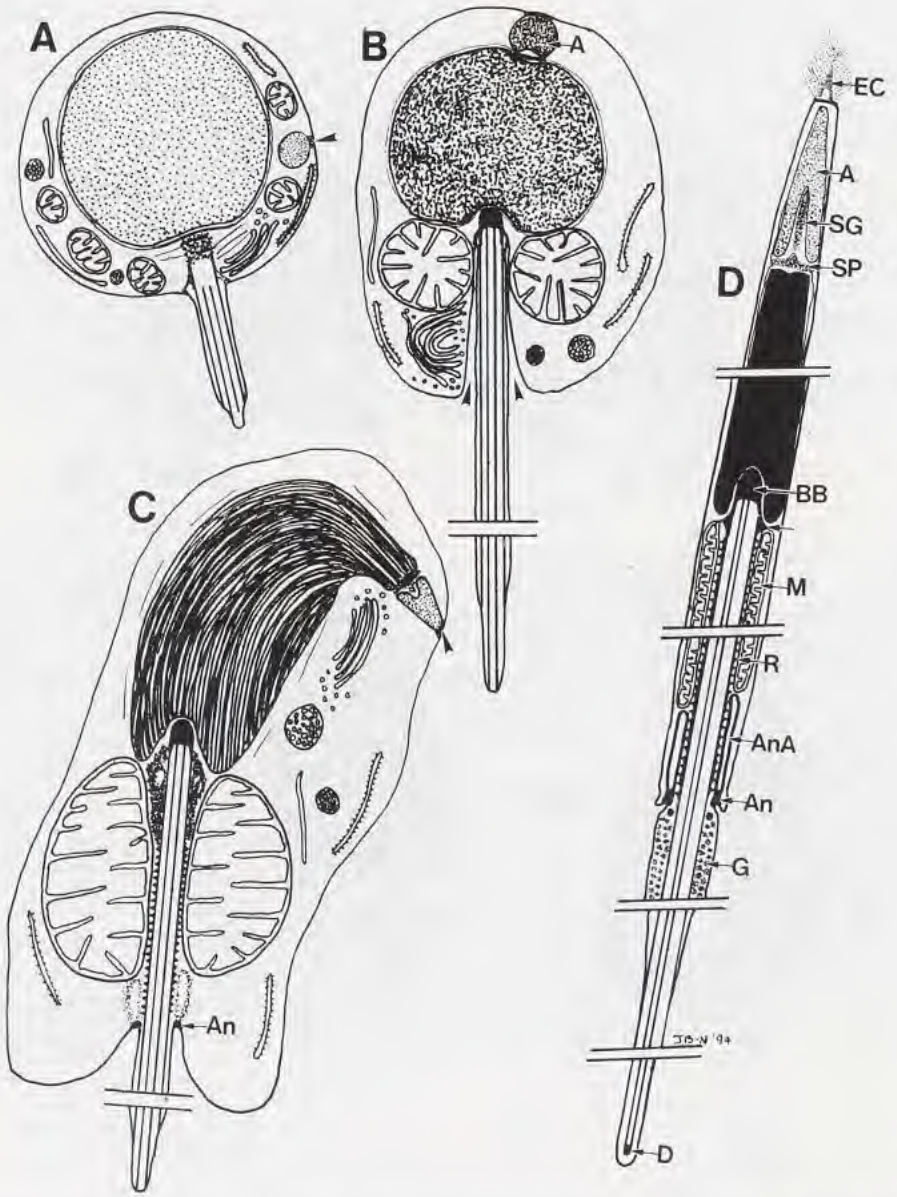


FIG. 38. — Spermiogenesis in *Epimenia australis*. **A**: Plesiosperm stage like that in Figs 36 & 37. Pro-acrosomal granule (arrowhead) is attached to plasma membrane by fibro-granular material. **B**: Pro-acrosome (A) is visible atop nucleus. Chromatin is granular but more tightly packed than in stage A. Mitochondria are fusing and aggregating at base of nucleus. Basal body, comprising fused proximal and distal centrioles, has migrated into centriolar fossa. Large active Golgi body is positioned next to temporary flagellar canal (arrowheads). **C**: Pro-acrosome (arrowhead) is attached to plasma membrane atop nucleus with Golgi body nearby. Chromatin has condensed into elongate fine fibres which are twisted inside nucleus. Annulus has broken away from distal centriole and is migrating posteriorly everting flagellar canal as it does so. Dense granular material surrounds apex of axoneme below basal body and a spiral ridge is evident posterior to this. Two large mitochondria are elongating posteriorly. **D**: Late spermatid with introsperm form. Extracellular striated cone (EC) is atop acrosome (A), which subtends acrosomal granule (SG) and is separated from dense nucleus by sub-acrosomal plate (SP). Basal body (BB) is housed in basal indentation of nucleus, blocked posteriorly by peri-basal body (arrow). Mitochondria (M) form an almost complete sheath around axoneme, reinforced by spiral ridge (R). Annulus (An) is in characteristic junction of mid-piece and glycogen piece and extends anteriorly as annulus adjuncts (AnA). Note also: Glycogen (G) and terminal density (D).

Source : MNHN, Paris

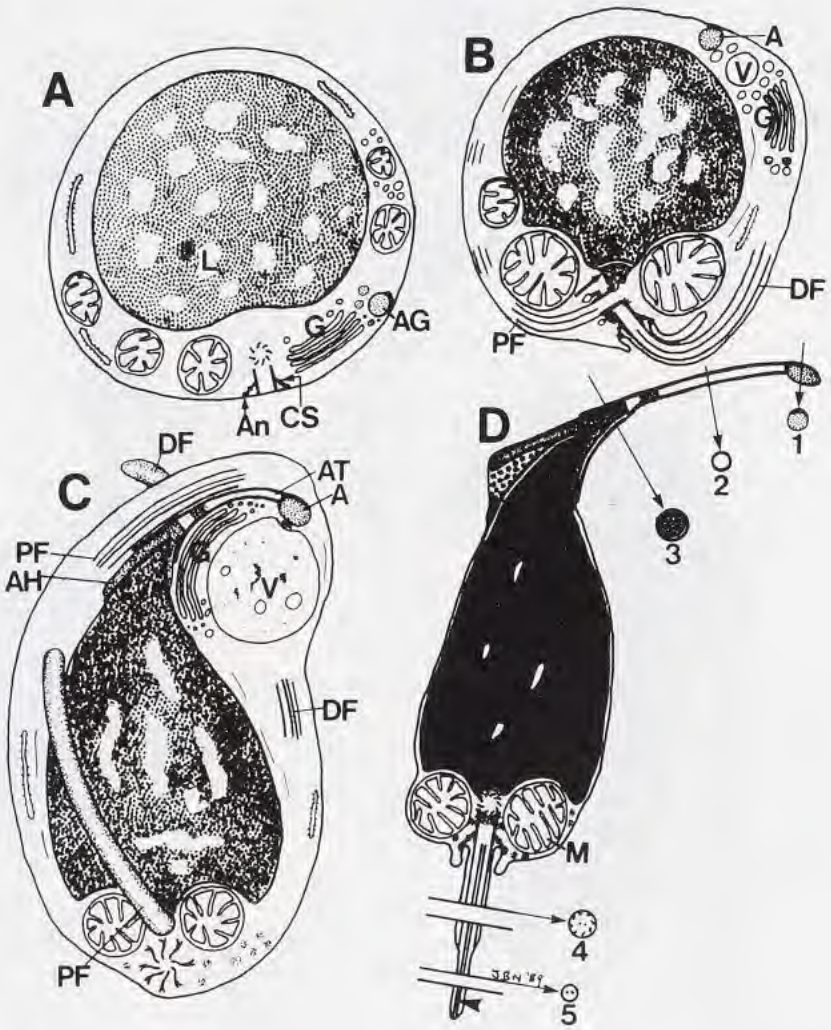


FIG. 39. — Spermiogenesis in *Chaetoderma argenteum*. (redrawn from [18]). **A**: Earliest part of stage A, showing distal centriole attached to plasma membrane by annulus (An) via centriolar satellite complex (CS) with nearby Golgi body (G) producing pro-acrosomal granule (AG). **B**: Proximal (PF) and distal flagella (DF) are visible. Mitochondria are grouped at base of nucleus; Golgi body (G) and proacrosome (A) have migrated anteriorly. Numerous secretory vesicles are fusing to form one larger vesicle (V). **C**: Proximal flagellum (PF) is aligned with apical tube (AT). The acrosome (A) at end of apical tube is connected to large vesicle (V) by fibro-granular material. Golgi body (G) is visible in an apical indentation of nucleus, adjacent to developing apical horn (AH) and apical tube. Distal flagellum exits anteriorly but will eventually encircle spermatid. **D**: Late spermatid showing homogeneously condensed nucleus with few lacunae. Spherical mitochondria (M) surround centrioles. Glycogen granules are visible near apical horn but are also sequestered in mid-piece. Cross sections 1-5: (1) acrosome; (2) apical tube; (3) apical horn; (4) flagellum; (5) end-piece.

Source : MNHN, Paris

Sperm-egg interaction in Chaetodermomorpha: The egg of *Chaetoderma*, regrettably, has not been observed with the scanning electron microscope. The only two ripe eggs observed with light microscopy, appeared to have a distinct hull with tiny bumps and small spinous projections (Figs 32, 33). However, at the moment there is no way to be certain that this resembles the hull of chitons, or is formed in a similar way. The mechanics of fertilization may be similar to chitons in that the acrosomal vesicle may digest a pore through the egg envelopes, followed by the apical tube penetrating down to the egg membrane. In both cases the perforatorium that characterizes other molluscs, and Metazoa in general, has been replaced by an anterior projection. In chitons this is a finite extension of the nucleus but in *Chaetoderma* the equivalent projection, the apical tube, is extensible. In response to egg water the apical tube elongated up to 1.5 times its original length, suggesting a novel polymerization process (Fig. 35). T.E.M. sections have not yet revealed what is changing in the anterior structures during elongation, but one suspects the polymerization of actin, which is almost universal among the eukaryotes. Elucidation of the details of sperm-egg interaction in *Chaetoderma* will have to await that elusive but opportune moment.

Phylogenetic analysis of Aculifera

Phylogenetic analysis (Tables 2-4) in MacClade, as well as "branch and bound" analysis in PAUP of 25 morphological characters (including new sperm/egg characters), produced a single minimum length tree (tree length = 67). The tree showed support for classification of three suborders of chitons, in two orders: Lepidopleurina in Lepidopleurida; Chitonina and Acanthochitonina in Chitonida. The Chitonina formed a clade based on synapomorphies for spinous hull projections that are narrow-based, posterior extension of midpiece altering sperm shape, as well as production of long coarse fibres in chromatin condensation. Of the families included in this study, Chitonina included: Callochitonidae, Chaetopleuridae, Acanthopleuridae, Chitonidae, Callistoplacidae, and Ischnochitonidae. Acanthochitonina are separable as a more recent clade by synapomorphies for mitochondrial arrangement in sperm, a fibrous complex in the sperm flagellum, and overall sperm shape, as well as adanal gills and broadly based hull cupules that are not spinous. Acanthochitonina included the families; Lepidochitonidae, Tonicellidae, Mopaliidae, and Acanthochitonidae. The Neomeniomorpha were used for outgroup comparison. The related Chaetodermomorpha clearly aligned with the neomenioids, in spite of different sperm types, based on synapomorphies for spiculate cuticle, distichous radula and other characters discussed below.

DISCUSSION

Phylogenetic relationships among Aculifera

Phylogeny of Aculifera currently is of great interest to malacologists because, although this group has generally been given a primitive status, recent morphological and molecular analyses place neomenioid aplacophorans basal to all extant Mollusca [67, 69].

Aplacophora (Neomeniomorpha and Chaetodermomorpha) are readily distinguished from Polyplacophora by the following synapomorphies: vermiform body with spiculate cuticle, distichous radula, reduced foot, gonads that open into the pericardium, and a cloaca-like posterior mantle cavity [66, 67, 69]. Also Chaetodermomorpha may be distinguished from Neomeniomorpha based on apomorphies for ectaquasperm structure, lack of seminal receptacles, presence of gills, as well as other characters [reviews: 66, 67, 69].

Source : MNHN, Paris

TABLE 2. — Data matrix and reconstructed states for internal nodes (Note: * = outgroup)

Node	1111111111222222 12345678901234567890123 45
Lepidopleuridae	00110114200100212110?11??
Chaetodermomorpha	121302141004?02010000???
Callochitonidae	23121114110211213111111 33
Chaetopleuridae	23221111110311214121111 22
Acanthopleuridae	23221111110321114122113 54
Callistoplacidae	2322111111?32111412111222
Chitonidae	2322111111?32111412211254
Ischnochitonidae	2322111111?32111412111143
Lepidochitonidae	33401112111232315121211 11
Tonicellidae	23401112120242315121211 11
Mopaliidae	23401112120242315131211 11
Acanthochitonida	2340111211?23231511331111
Neomeniomorpha*	00000000000000000000000 00
1	00100014100000200000000 00
2	00100114100100212110111 11
3	23101114110211213111111 11
4	23121114110211213111111 22
5	23221111110311214121111 22
6	23221111110321114121111 22
7	23221111110321114121112 22
8	23221111110321114122112 54
9	23401112110232315111211 11
10	23401112110232315121211 11
11	23401112120242315121211 11

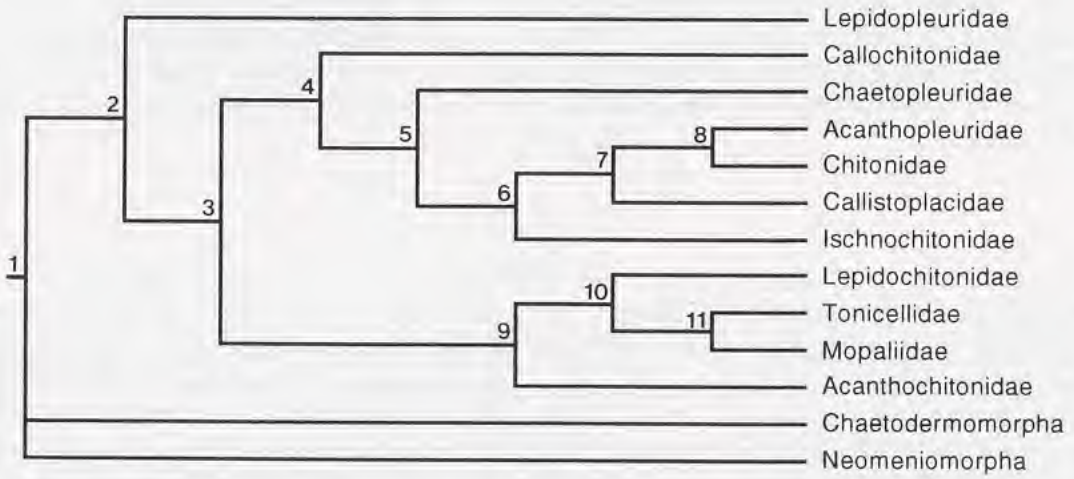


FIG. 40. — Single most parsimonious tree resulting from branch and bound analysis in PAUP of the data matrix in Table 2. Branch and bound search settings were, initial upper bound: unknown (compute via stepwise); addition sequence: furthest; no multistate taxa; unrooted trees rooted using outgroup method. Character state optimization: accelerated transformation (ACCTRAN); tree length = 67; consistency index = 0.970; homoplasy index = 0.030; retention index = 0.967; HI excluding uninformative characters = 0.033; rescaled consistency index = 0.939. The ingroup (1) and all interior nodes (2-11) are labelled. The apomorphy hypotheses supporting each interior node are shown in Table 3.

Source : MNHN, Paris

TABLE 3. — Apomorphy hypotheses for internal nodes of cladogram of Aculiferan phylogeny (Fig. 40). Unless otherwise stated character changes are from 0→1.

Node 1→2:	6,12,16,17(0→2),18,19,21,22,23,24,25
Node 2→3:	1(0→2),2(0→3),5,10,12(1→2),13,14,17(2→3),20
Node 3→4:	4(0→2),24(1→2),25(1→2)
Node 4→5:	3(1→2),8(4→1),12(2→3),17(3→4),19(1→2)
Node 5→6:	13(1→2),15(2→1)
Node 6→7:	23(1→2)
Node 7→8:	20(1→2),24(2→5),25(2→4)
Node 3→9:	3(1→4),8(4→2),13(1→3),14(1→2),15(2→3),17(3→5),21(1→2)
Node 9→10:	19(1→2)
Node10→11:	10(1→2),13(3→4)

TABLE 4. — Characters used in cladistic analysis of Aculifera with character state codes. See Table 2 for data matrix.

Sperm and Egg Data

(1). ACROSOME: 0: cone with subacrosomal plate (SA.P.); 1: simple vesicle with SA.P.; 2: complex vesicle with SA.P; 3: simple vesicle with SA.P.

(2). SUBACROSOMAL GRANULE: 0: loose granular; 1: rod granular; 2: apical horn & tube; 3: absent

(3). MITOCHONDRIAL ASSOCIATIONS: 0: mitochondria (M) fused unspiralled; 1: M in ring around central proximal and distal centrioles (PC/DC); 2: offset PC/DC with basal M; 3: offset PC/DC associated with basal not lateral M; 4: offset PC/DC associated with basal & lateral M.

(4). CHROMATIN CONDENSATION: 0: develop fine long fibres; 1: develop fine then coarse short fibres; 2: develop fine then coarse long fibres; 3: "granular" chromatin.

(5). GOLGI BODY PRODUCTION OF ACROSOME: 0: proacrosomal granule migrates on plasma membrane; 1: proacrosomal vesicles migrate, fuse; 2: proacrosomal granule migrates with Golgi body.

(6). GLYCOGEN RESERVES: 0: stored in tail; 1: stored in midpiece; 2: stored in anterior & midpiece.

(7). FLAGELLAR CANAL: 0: present but temporary, annulus breaks from D.C.; 1: absent.

(8). FLAGELLUM REINFORCED: 0: thickening around axoneme; 1: unilateral thickening of plasma membrane; 2: unilateral fibrous body; 3: absent.

(9). FLAGELLUM TERMINATION: 0: 2 central microtubules elongate+density; 1: 2 centrals, short; 2: 9+2 configuration.

(10). FERTILIZATION SITE: 0: anywhere on egg; 1: between hull projections; 2: inside hull projections.

(11). HULL PORES: 0: absent; 1: present

(12). SPERM HEAD SHAPE: 0: filamentous; 1: oval ; 2: oval w. nuclear filament; 3: oval w. offset nuclear filament; 4: oval w. apical tube.

(13). HULL TYPE: 0: smooth; 1: complex spines; 2: simple spines w. modified tips; 3: closed cupules; 4: open cupules.

(14). CUPULE BASE SIZE: 0: absent; 1: 5-30 µm; 2: 50-90 µm [71]

(15). MITOCHONDRIA: 0: 1-2; 1: 2-4; 2: 4-6; 3: 6-8.

Shell Valve [28,78] and Gill Placement Data [71]

(16). EXTERNAL COVERING: 0: spiculate cuticle; 1: 8 shell valves; 2: smooth.

(17). GILLS: 0: absent; 1: 2 ctenidia posterior; 2: Type 1 *ad*anal; 3: Type 2 *ad*anal; 4: Type 3 *ad*anal; 5: Type 4 *ab*anal.

(18). ARTICULEMENTUM: 0: absent; 1: present.

(19). CHITON VALVE MORPHOLOGY: 0: absent; 1: modern valve (V) shape; 2: multifissurated terminal Vs 3: post. V. w. post. median sinus; 4: girdle & post.V. fissured.

(20). INSERTION PLATES: 0: absent; 1: slitted; 2: pectinated; 3: reduced slits.

(21). GIRDLE ORNAMENTATION: 0: absent; 1: girdle elements similar; 2: potential bristles; 3: girdle fissured; 4: sutural tufts.

(22). RADULA MORPHOLOGY: 0: distichous; 1: docoglossan.

(23). PHOTORECEPTORS: 0: absent; 1: macro & micro aesthetes; 2: intrapigmental aesthetes; 3: ocelli.

(24). GILLS FROM NEPHROPORE: 0: absent; 1: 1-2 gills; 2: 3-4; 3: 5-6; 4: 7-8; 5: >9

(25). GILLS FROM GONOPORE: 0: absent; 1: 1-3 gills; 2: 4-6; 3: 7-9; 4: >10.

Source : MNHN, Paris

Egg hull projections and associated follicle cells are synapomorphic to chitons, uniting the entire group. Furthermore, all chitons more recent than lepidopleurids are united by having an elongate sperm nuclear filament with reduced acrosome, cupular hull projections, slitted insertion plates and modern gill placement. This evidence supports the theory of THIELE [75] that chitons are a monophyletic group derived from a lepidopleurid-like ancestor, and argues strongly against a polyphyletic origin as proposed by ASHBY [5]. Historically, Polyplacophora have been difficult to resolve because potentially useful characters such as radula morphology, which have an extensive fossil record, are virtually unchanged in the chitons [72]. Other characters, such as girdle morphology or hull spine tip structure, are highly variable and may have arisen independently, or may be polymorphic for a single taxon [28, 71]. EERNISSE [28] first suggested using placement of gills, sperm morphology and egg hull data as a way to develop independent character sets to test the validity of previous phylogenies based on shell valve morphology alone [reviews: 72, 78].

The addition of a new character set based on sperm morphology and mode of fertilization, presented here for the first time, has enabled a preliminary reassessment of chiton phylogeny. The present analysis combined sperm and egg hull data with gill placement and morphology [71], as well as shell valve morphology [28, 78]. The single minimum length tree showed strong support for retaining three suborders of chitons, Lepidopleurina, Chitonina, and Acanthochitonina [71]. Acanthochitonina are more derived than Chitonina based on the sperm fibrous complex, adanal gills and broadly based hull projections. Lepidochitonids clearly are included in Acanthochitonina by possessing each of these synapomorphic characters. Previous classifications based largely on shell valve morphology have given lepidochitonids a rather primitive status placing them between lepidopleurids and other chitonids [reviews: 72, 78]. The present analysis reinstates lepidochitonids in their own family, Lepidochitonidae, as they clearly fall outside the Tonicellidae (based on apomorphies for hull pores, simplified acrosome, and cone-like hulls). This disagrees with a recent classification that placed lepidochitonids within Tonicellidae, but supports the inclusion of both Lepidochitonidae and Mopaliidae within the superfamily Tonicelloidea [71].

The Chitonina have been more difficult to resolve. However, the present analysis provides support for maintaining the suborder Chitonina, as suggested by SIRENKO [71], rather than Ischnochitonina [72, 78]. The superfamily grouping Ischnochitonoidea Dall, 1889 [71], in this analysis included the families Chaetopleuridae, Acanthopleuridae, Chitonidae, Callistoplacidae, and Ischnochitonidae, but excluded the Callochitonidae. Relaxing parsimony by one tree length (= 68) creates an even more paraphyletic assemblage and underscores the need for more synapomorphic characters, particularly for this suborder. More detailed analyses of oogenesis and spermiogenesis (such as acrosome substructure and hull deposition) may provide additional characters that will enable better resolution of this complex taxon.

Phylogenetic relationship between Aculifera and other Bilateria: quest for the origin of internal fertilization

FRANZÉN's hypothesis that the biology of fertilization correlates with sperm morphology works well in most situations [33, 34]. Furthermore, the idea that the "primitive sperm" [33] or "plesiosperm" [45], (which is found throughout the Radiata), represents the primitive condition in basal Metazoa, is not questioned by most biologists. This is partly because sperm of more recent groups usually pass through a stage in spermiogenesis that resembles the plesiosperm type, but also because recent molecular and morphological analyses indicate a monophyletic origin of Metazoa from a diploblastic ancestor [30, 50]. However, the idea that ectaquasperm generally represent the primitive condition wherever they occur in more recent groups [8, 9, 33, 34, 44], is actively debated [22, 23, 38, 46, 47, 54, 60]. For example, JAMIESON [46, page 2] discussed this topic in his recent book on fish evolution: "The term plesiosperm recognizes that, whether or not aquasperm have evolved in some sections of the Metazoa from more complex sperm, the

Source : MNHN, Paris

"primitive" sperm facies may be genuinely plesiomorphic in many metazoan groups. This is not to exclude from further investigation the possibility (a) that the earliest metazoans may have been free-spawning with sperm of an even simpler form than the plesiosperm or (b) that they were internally fertilizing with more or less complex sperm".

It was once said that "it is unlikely that primitive spermatozoa from different animal phyla should have evolved independently on several occasions obtaining the same morphology by convergence."[9]. How much more unlikely is it that primitive members of at least twenty animal phyla have evolved a basically similar, infinitely more complex introsperm by convergence? It would seem more logical to assume secondary evolution of the simpler aquasperm from the more complex introsperm. Recent molecular [62] and morphological [67, 69] analyses of neomenioids place them basal to all extant Mollusca. It was surprising to find how typical is the introsperm of neomenioids [22, 23]. It is similar in basic structure and arrangement of organelles to introsperm of primitive members of most bilaterian phyla, including some nemertines [2], bryozoans and entoprocts [35], arthropods [3], annelids [53] as well as some deuterostomes [review: 46]. In particular, neomenioid introsperm closely resemble parasperm of protodrilids [53], a primitive annelid group, formerly included in the now abandoned Archiannelida. The above evidence provides support for current theories that recognize Eutrochozoa (animals with trochophore larvae) as a distinct clade [30, 37, 50], which includes nemertines [76] and perhaps some other invertebrate taxa. Besides the striking similarity to protodrilid parasperm, introsperm of neomenioids have much in common with introsperm of Neritimorpha [15] and "primitive" Caenogastropoda [13, 40], suggesting that current ideas on gastropod evolution [39] also may require revision. Perhaps the Patellogastropoda, presently considered basal to Gastropoda [39], diverged early from a neritimorph-like ancestor?

All Platyhelminthes have introsperm but until the key paper on the primitive turbellarian *Nemertoderma* [77] demonstrated the typical introsperm pattern with a plausible acrosome, rodlike nucleus, elongate midpiece, and single typical flagellum, it had been easy for scientists to dismiss the sperm of Platyhelminthes as highly derived, which most of them are [41]. Since then it has become obvious that introsperm are basal at least to the Platyhelminthomorpha (Platyhelminthes and Gnathostomulida) [6, 73]. Moreover, the above evidence strongly suggests that introsperm are basal to all Bilateria [22, 23]. If true, this would unite Eubilateria and Platyhelminthomorpha as a monophyletic clade.

The basal Platyhelminth groups (Nemertodermatida, Catenulida, Acoela and Macrostomida) are minute organisms, attaining only a few millimeters in length and fractions of a millimeter in width [6]. AX suggests that this is because they all possess the plesiomorphic condition of a simple mouth pore, which limits nutrient uptake and hence body size [6]. More importantly it leaves only minute spaces between the endoderm and ectoderm with little room for expansion of gonads. The corresponding small size of basal Gnathostomulida suggests that the bilaterian stem was a microscopic organism [6]. Based on trace fossil evidence the earliest Bilateria are considered to have emerged as minute wormlike organisms in the Proterozoic, between 600 M and 1000 Myrs ago [25, 26]. BOADEN [11, 12] agreed with this and formulated the "Thyobios" hypothesis which suggested that tiny anaerobic Bilateria evolved in shallow water, anoxic sediments during the earliest emergence of the Metazoa. He suggested that as a consequence of their small size they would have fertilized internally [12]. This correlates with geological evidence of low atmospheric oxygen concentrations until the middle Vendian (550 Myrs) [27, 49]. A late Proterozoic increase in atmospheric oxygen coincides with the emergence of macroscopic Metazoa, first evident in the Ediacaran fauna [25, 27, 49]. However, the trace fossil evidence indicates that Bilateria were well established before this time, and key ichnogenera are traceable through all the early biozones [26].

Furthermore, the suggestion that free spawning always reflects primitive status [33, 44] does not withstand close scrutiny [38, 54]. A study of extant marine invertebrates has shown that reproductive biology (including fertilization biology) depends on a series of covariable

Source : MNHN, Paris

reproductive traits [54]. When body length is less than 1mm metazoans fertilize internally with introsperm; only larger bodied forms successfully free-spawn ectaquasperm. Much of the reason for this probably relates to fertilization success. Recent studies have shown that among free-spawners, selection favours larger sperm numbers, larger egg numbers, as well as larger egg size [51, 52]. Other key factors contributing to success include distance between mates, and spawning synchronicity [7, 10, 51, 57]. Tiny triploblastic organisms would not be able to expand the gonads to produce viable numbers of sperm and eggs for free-spawning and selection would favour some form of internal fertilization [54], or hermaphroditism [36].

If it is true that introsperm were basal to Bilateria, then ectaquasperm must have been secondarily developed many times. How could this have occurred? As mentioned, sperm biologists are well aware that all sperm pass through a stage in their ontogeny that resembles the plesiosperm [33, review:47]. Perhaps the re-expression of the plesiosperm form has occurred by truncation of spermiogenesis, a type of progenesis? This has been advanced to explain the presence of plesiomorphic structures in the sperm of schistosomes [48], and probably explains secondary development of ectaquasperm in teleosts [47], as well as sabellid polychaetes [60]. If progenetic spermiogenesis has occurred frequently among animal phyla, we might expect a relatively simple mechanism to control it, perhaps a molecular switch. In vertebrates completion of spermiogenesis is dependent on Sertoli cells [1]. Recently, a regulatory protein was isolated from the round spermatid stage of rats (≈ plesiosperm stage), which was capable of turning on the production of Sertoli cell total protein and transferrin secretion [55]. If these proteins are found to play key roles in the completion of spermiogenesis, the regulatory protein produced by the spermatids could be the kind of molecular switch we are looking for. In the absence of such a regulatory protein, spermiogenesis could be truncated at the plesiosperm stage.

If secondary expression of ectaquasperm has occurred frequently, as suggested, there must be a selective pressure favouring this type of sperm in external fertilization. Evidence indicates that introsperm of some marine invertebrates are capable of swimming effectively in sea water [14,60]. This suggests that the selective advantage to progenetic spermiogenesis may relate more to lower energetic costs and shorter generation times in producing the less complex ectaquasperm, rather than to more efficient locomotion in a sea water medium.

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Source : MNHN, Paris

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Source : MNHN, Paris

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Source : MNHN, Paris

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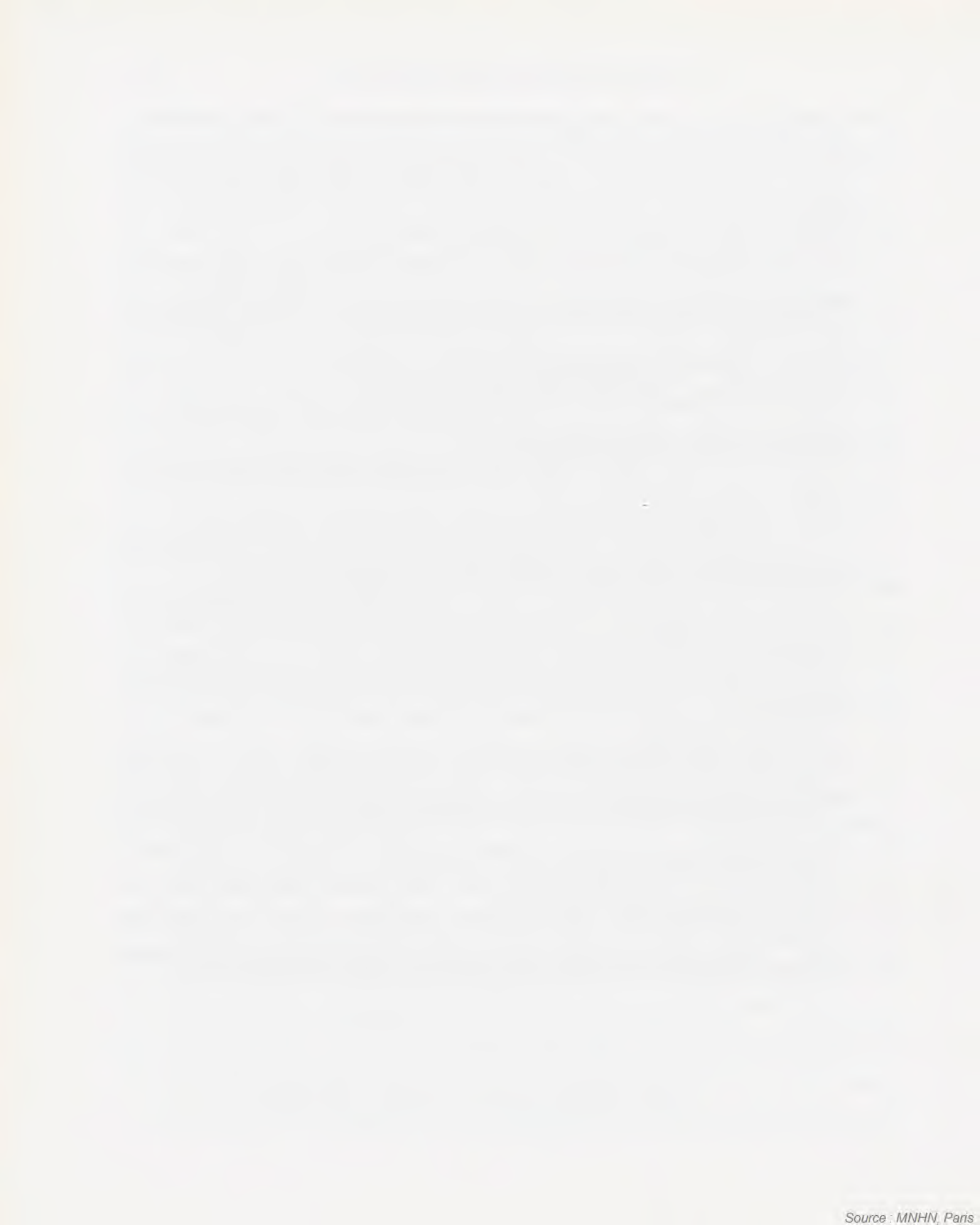
Source : MNHN, Paris



Source : MNHN, Paris

Comparative Spermatozoal Ultrastructure and its Taxonomic and Phylogenetic Significance in The Bivalve Order Veneroida

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ABSTRACT

A comparative study of sperm ultrastructure in the Veneroida, an ecologically and economically important order of bivalve molluscs, has revealed significant differences between taxa. Most veneroid spermatozoa are of the aquasperm type (general features: conical acrosome, nucleus usually short, midpiece consisting of a ring of round mitochondria surrounding the centrioles, single simple flagellum). Five principle sperm morphologies can be recognized in the Veneroida, these correlating to varying degrees of precision with groups of superfamilies. Group A (Lucinoidea, Cardioidea (including Tridacnidae), Veneroidea, Mactroidea, Chamoidea, Solenoidea, Tellinoidea (one species of Donacidae)): basal ring of acrosome without visible substructure and not developed longitudinally; nucleus curved if rod-shaped. Group B (Galeommatoidea (*Mysella*, *Scintilla*, *Divariscintilla*)): sperm similar to Group A and especially C, but with strongly tilted acrosomal complex and basal ring developed transversely. Group C (Tellinoidea (Donacidae, Tellinidae), Arcticoidea, Dreissenoidea, Galeommatoidea (*Lasaea*)): basal ring developed longitudinally, sometimes showing substructure; overlap between mitochondria and nucleus only in Tellinidae. Group D (Tellinoidea (Scrobiculariidae), Corbiculoidea): acrosome and nucleus elongate; pronounced overlap of mitochondria with nucleus. Group E (Carditoidea + Crassatelloidea assemblage): acrosome and nucleus elongate; midpiece exhibiting usually 8 mitochondria; proximal centriole modified into well developed rootlet. The widespread occurrence of the Group A spermatozoon within the Veneroida including the basal superfamily Lucinoidea, suggests that this sperm type was typical of early veneroids. In contrast, the sperm morphologies encountered in Groups D and E are unknown elsewhere within the Bivalvia, and undoubtedly represent modifications from a less complex sperm type (e.g. Group A sperm).

RÉSUMÉ

Ultrastructure comparée des spermatozoïdes et sa signification taxonomique et phylogénétique dans l'ordre des Veneroida (Bivalves)

L'étude comparée de l'ultrastructure des spermatozoïdes chez les Veneroida, un groupe de Mollusques important du point de vue écologique et économique, a montré des différences significatives entre les taxons. La plupart des spermatozoïdes des Veneroida sont du type aquaspermatozoïde, dont les caractéristiques générales sont un acrosome conique, un noyau généralement court, une pièce intermédiaire consistant en un anneau de mitochondries rondes entourant les centrioles et un flagelle simple et unique. Cinq morphologies principales de spermatozoïdes peuvent être reconnues chez les Veneroida, et peuvent être corrélées avec des degrés variés de précision avec les groupes ou les superfamilles. Groupe A (Lucinoidea, Cardioidea (y compris les Tridacnidae), Veneroidea, Mactroidea, Chamoidea, Solenoidea, Tellinoidea (une espèce de Donacidae)): anneau basal de l'acrosome sans substructure visible et non développé longitudinalement; noyau courbe si en

HEALY, J. M., 1995. — Comparative spermatozoal ultrastructure and its taxonomic and phylogenetic significance in the bivalve order Veneroida. *In*: JAMIESON, B. G. M., AUSIO, J., & JUSTINE, J.-L. (eds), Advances in Spermatozoal Phylogeny and Taxonomy. *Mém. Mus. natn. Hist. nat.*, **166** : 155-166. Paris ISBN : 2-85653-225-X.

Source : MNHN, Paris

forme de baguette. Groupe B (Galeommatoidea (*Mysella, Scintilla, Divariscintilla*)): spermatozoïde similaire au groupe A et spécialement au groupe C, mais avec un complexe acrosomien fortement incliné et un anneau basal développé transversalement. Groupe C (Tellinoidea (Donacidae, Tellinidae), Arcticoidea, Dreissenoidea, Galeommatoidea (*Lasaea*)): anneau basal développé longitudinalement, montrant parfois une substructure; chevauchement entre les mitochondries et le noyau seulement chez les Tellinidae. Groupe D (Tellinoidea (Scrobiculariidae), Corbiculoidea): acrosome et noyau allongés; chevauchement prononcé des mitochondries et du noyau. Groupe E (Carditoidea + assemblage des Crassatelloidea): acrosome et noyau allongés; pièce intermédiaire montrant généralement huit mitochondries; centriole proximal modifié en une racine bien développée. La présence très répandue du spermatozoïde du groupe A chez les Veneroida, y compris dans la superfamille primitive Lucinoidea, suggère que ce type de spermatozoïde était typique des premiers Veneroida. Au contraire, les morphologies de spermatozoïdes rencontrées dans les groupes D et F sont inconnues dans d'autres groupes des Bivalvia, et représentent de manière certaine des modifications à partir d'un type de spermatozoïde moins complexe (par exemple le spermatozoïde du groupe A).

The Veneroida constitute one of the most important extant orders of bivalve molluscs. Included within the group are several marine families of economic and ecological significance such as the cockles and giant clams (Cardiidae, Tridacnidae), venus shells (Veneridae and allies), tellins (Tellinidae and allies) and the estuarine/ freshwater family Corbiculidae. Because of this, studies of veneroid reproductive biology are of considerable importance in understanding the success of the order. In addition, comparative work on sperm fine structure in this and other molluscan groups continues to generate characters of taxonomic and phylogenetic significance, most notably in the internally fertilizing Gastropoda which have complex, often polymorphic, spermatozoa [12, 13, 17, 25, 26]. Although sperm morphology has been examined for several bivalve species, it is only in recent years that a comparative approach has been applied within this class at the ultrastructural level. In 1971 GHARAGOZLOU-VAN GINNEKEN & POCHON-MASSON [10] were the first authors to demonstrate significant differences between species and genera within the Veneridae (Veneroidea, Veneroida, see Table 1). Similar studies have been carried out by Hodgson and co-workers [19-21] on various Veneroida (congeneric species of Solenidae and Donacidae, see Table 1) and Mytiloidea. Such information is not only useful in verifying the validity of species, but also offers an opportunity to test their relationships to other congeners - the latter exercise being dependent on the availability of other sperm data. On a broader scale, sperm ultrastructural studies by POPHAM [41] and HEALY [14, 17] have also been directed towards investigating the taxonomic and evolutionary relationships between bivalve subclasses and orders.

Above the species and genus levels, sperm ultrastructure appears to be a promising source of taxonomic and phylogenetic information in the Veneroida based on my own observations (presented here, see Table 1) as well as data from published accounts (Table 2). The present study examines comparative sperm morphology among veneroid bivalves and concludes with a discussion of the possible taxonomic and phylogenetic implications of all available data for the group. Although some authors exclude the Lucinoidea from the Veneroida (placed in a separate order by MORRIS [33], in a separate subclass by POJETA [40]), I have adopted the more traditional approach [1, 5, 32, 49] and retained it within the order.

MATERIAL AND METHODS

The present survey of veneroid sperm ultrastructure is based on the author's observations (using species collected from the Queensland coast: see Table 1) combined with information already available in the literature (see Table 2). For most species investigated herein, fixation of testicular tissue was carried out using ice cold (0-4°) glutaraldehyde (3.5% in 0.1 M phosphate buffer containing 10% w/v sucrose) followed by placing tissue pieces into 1% osmium tetroxide (at 0-4°C, in 0.1 M sucrose adjusted phosphate buffer), followed by three rinses in phosphate buffer (at 0-4°C, buffer sucrose adjusted), ethanol dehydration and embedding in Spurr's epoxy resin. For *Codakia punctata* (Lucinoidea) and *Tellina rostrata* (Tellinoidea) sea water formalin testis tissues only were processed for TEM. Semithin and ultrathin sections were cut using an LKB UM IV Ultrotome stained according to the procedure of DADDOW [7] and examined using an Hitachi H-300 transmission electron microscope.

Source : MNHN, Paris

TABLE 1. —Veneroid taxa examined in the present study and dimensions of acrosome, nucleus and midpiece (dimensions averaged, n = 5). ND = not determined. Dimensions expressed in µm. L = maximum length; D = maximum diameter. Maximum diameter measurements for acrosomal vesicle and nucleus taken through base of these sperm components.

Taxa	Acrosomal Vesicle (LxD)	Nucleus (LxD)	Midpiece (LxD)
Superfamily Lucinoidea			
Family Lucinidae			
Codakia punctata	0.50 x 0.95	3.68 x 1.20	1.00 x 1.40
Superfamily Cardioidea			
Family Cardiidae			
Fragum hemicardium	0.25 x 0.60	2.16 x 1.55	0.68 x 1.75
Fragum unedo	0.27 x 0.67	2.20 x 1.35	0.53 x 1.70
Acrosterigma reeveanum	0.34 x 0.46	ND	ND
Family Tridacnidae			
Tridacna maxima	0.50 x 0.70	ND	ND
Superfamily Mactroidea			
Family Mactridae			
Spisula trigonella	0.44 x 0.38	1.25 x 2.00	1.00 x 2.25
Superfamily Veneroidea			
Family Veneridae			
Placamen calophyllum	0.73 x 0.46	3.06 x 1.00	0.53 x 0.70
Circe cf *plicatina*	0.64 x 0.74	3.0 x 1.35	0.64 x 1.70
Paphia sp.	0.67 x 0.56	1.8 x 1.52	0.60 x 1.60
Family Glauconomidae			
Glauconome sp.	1.20 x 0,37	4.50 x 0.75	1.66 x 0.66
Superfamily Tellinoidea			
Family Donacidae			
Donax deltoides	1.18 x 0.88	0.67 x 0.75	0.60 x 1.75
Family Tellinidae			
Tellina rostrata	0.55 x 0.45	0.00 x 0.70	1.56 x 0.82
Superfamily Arcticoidea			
Family Trapeziidae			
Trapezium sublaevigatum	0.45 x 0.56	2.00 x 1.20	0.46 x 1.36

Source : MNHN, Paris

TABLE 2. — Veneroid taxa previously investigated for sperm ultrastructure

Superfamily Cardioidea	
Family Cardiidae	
Fulvia tenuicostata	[41]
Cardium edule	[46]
Superfamily Mactroidea	
Family Mactridae	
Spisula solidissima	[22, 29]
Superfamily Veneroidea	
Family Veneridae	
Venerupis aurea	[10]
Venerupis corrugata	[10]
Ruditapes decussatus	[10, 39]
Tapes rhomboides	[10]
Callista chione	[34]
Tivela polita	[43]
Superfamily Solenoidea	
Family Solenidae	
Solen cylindraceus	[21]
Solen capensis	[21]
Ensis ensis	[4]
Superfamily Chamoidea	
Family Chamidae	
Chama macerophylla	[22]
Superfamily Tellinoidea	
Family Donacidae	
Donax madagascariensis	[20]
Donax sordidus	[20]
Donax serra	[20]
Donax trunculus	[47]
Family Scrobiculariidae	
Scrobicularia plana	[48]
Superfamily Dreissenoidea	
Family Dreissenidae	
Dreissena polymorpha	[9]
Superfamily Galeommatoidea	
Family Galeommatidae	
Lasaea subviridis	[35, 38]
Mysella tumida	[35, 36]
Pseudopythina rugifera	[37]
Divariscintilla yoyo	[8]
Divariscintilla troglodytes	[8]
Scintilla sp.	[8]
Superfamily Corbiculoidea [13]	
Family Corbiculidae (*Corbicula*)	
Corbicula sandai	[11]
Corbicula fluminea	[28]
Superfamily Carditoidea	
Family Carditidae	
Cardita muricata	[18]
Superfamily Crassatelloidea	
Family Crassatellidae	
Eucrassatella cumingii	[18]
Eucrassatella kingicola	[18]
Talabrica aurora	[18]

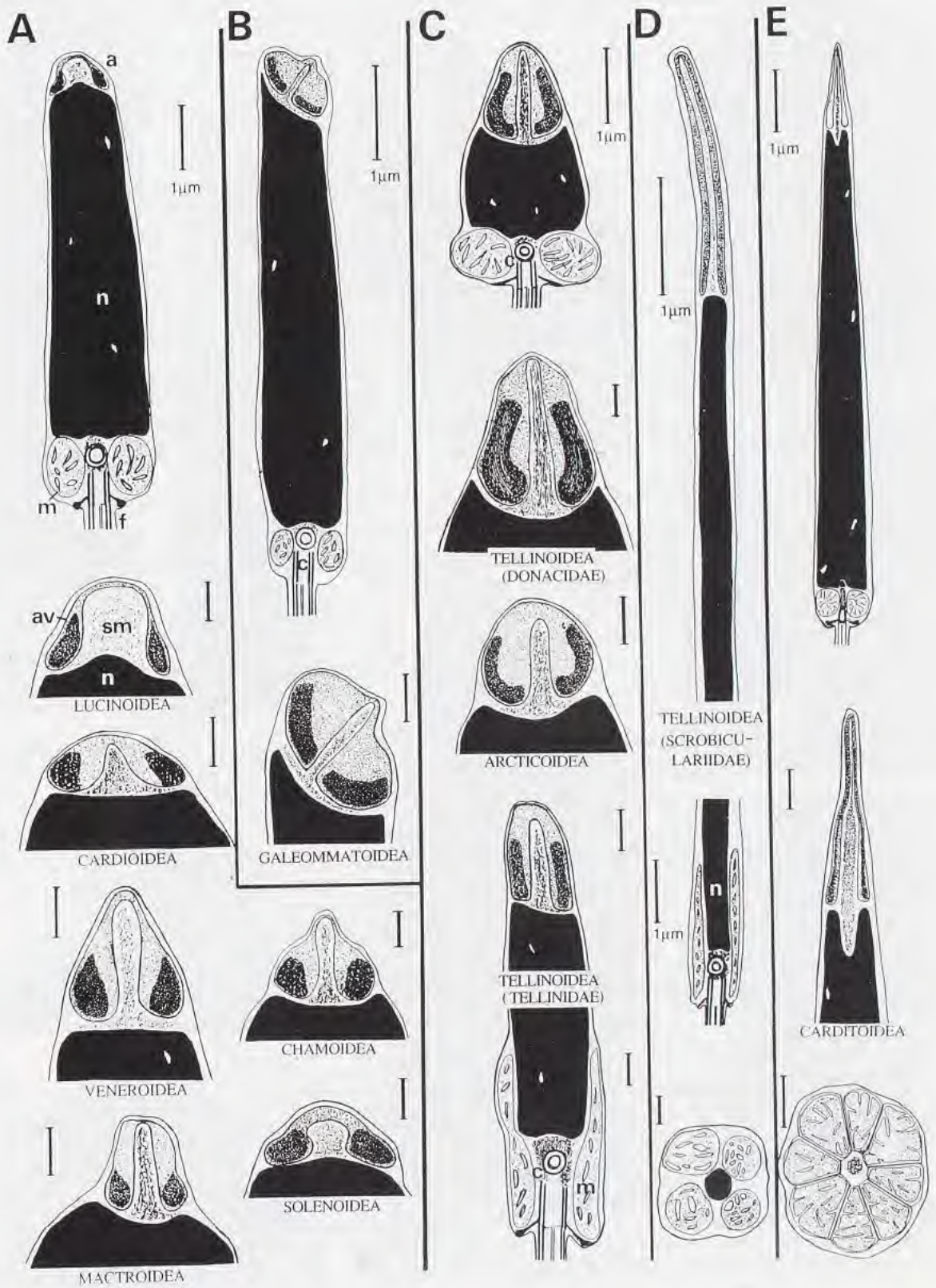
RESULTS AND DISCUSSION

Aquasperm features of veneroid spermatozoa

Most veneroid spermatozoa, like those of the majority of other bivalve taxa, could be classed as unmodified or relatively unmodified aquasperm, the principal features of which are as follows: (1) a well developed, conical acrosomal vesicle; (2) a short or relatively short nucleus (with electron-lucent lacunae); (3) a short midpiece (containing round mitochondria, usually four or five in number, surrounding a pair of orthogonally arranged, triplet substructure centrioles); (4) a radial array of satellite fibres anchoring the distal centriole (basal body) to the plasma membrane; (5) a simple flagellum (9+2 axoneme sheathed only by the plasma membrane). Although most differences in sperm morphology between veneroid taxa involve acrosomal and/or nuclear

FIG. 1. — **A-E:** The five major sperm morphologies occurring within the Veneroida. a, acrosomal complex; av, acrosomal vesicle; c, centrioles (proximal and distal); f, flagellum; m, mitochondria (of midpiece); n, nucleus; sm, subacrosomal material. Scale bars = 0.25 µm, except where indicated. Sources of data: present study except Chamoidea [2]; Solenoidea [4]; Galeommatoidea [5, 6]; Tellinoidea - Scrobiculariidae [9].

Source : MNHN, Paris



Source : MNHN, Paris

features, some useful variations in midpiece architecture are also apparent such as differing mitochondrial number and presence/absence of an unmodified proximal centriole. The five main sperm morphologies occurring in the Veneroida, and their chief characteristics, are as presented below (taxa studied here indicated by *, other sources referenced by number).

Group A: Lucinoidea [*], Cardioidea [, 46], Veneroidea [*, 10, 20, 34, 39, 43], Mactroidea [*, 22, 29], Tellinoidea (Donacidae* - Donax trunculus *only, [47]), Chamoidea [22], Solenoidea [4, 21] (Figs 1A, 2A-E)*

General features: (1) acrosomal vesicle short, conical, deeply invaginated, with thick, highly electron-dense basal ring (longitudinal profile of ring round to pyriform); subacrosomal material either diffuse or with axial rod differentiated; (2) nucleus short or rod-shaped (usually curved if rod-shaped) depending on family or genus, typically with no or only a poorly developed apical depression (apex often convex); (3) midpiece with two unmodified (triplet substructure) centrioles surrounded by four or most commonly five rounded mitochondria; (4) satellite fibre complex anchoring distal centriole to plasma membrane; (5) single flagellum with 9+2 axoneme.

The widespread occurrence of this sperm morphology within the Veneroida, including the Lucinoidea (considered by many authors as the oldest extant veneroid superfamily), suggest that it was probably characteristic of the earliest members of the order. Variation in the shape and dimensions of the acrosomal vesicle and nucleus is considerable in the Veneroidea.

*Group B: Galeommatoidea (*Lasaea, Scintilla, Divariscintilla*) [8, 35]. (Fig. 1B)*

Features as for Groups A and especially C, but differing in having the acrosomal complex arranged at a considerable angle to the longitudinal axis of the spermatozoon. Basal ring crescentic (as in Group C) but developed transversely.

This type of sperm morphology was probably derived from the more widespread Group C type, through re-alignment of the acrosomal vesicle (and its crescentic basal ring). A detailed study of *Scintilla* and *Divariscintilla* [8] shows that the tilted positioning of the acrosomal vesicle seen in mature spermatozoa takes place in the final phase of spermiogenesis.

Group C Tellinoidea (Donacidae [20,], Tellinidae [*]), Arcticoidea [*], Dreissenoidea [9], Galeommatoidea (*Mysella *[35]). (Figs 1C, 2F-I)*

Features resembling those of Groups A and especially B. Group C acrosomes differs from those of Group B in not being tilted and in having the basal ring developed longitudinally rather than transversely. Substructure sometimes visible within the acrosomal vesicle contents. Marked overlap of midpiece and nucleus in *Tellina* (Tellinidae, Tellinoidea) similar to Group D.

Group D: Tellinoidea (Scrobiculariidae [48]), Corbiculoidea [11, 28]. (Fig. 1D)

Acrosomal vesicle slender, almost totally invaginated with basal ring not clearly defined. Subacrosomal material diffuse, without axial rod. Nucleus elongate and slender. Midpiece

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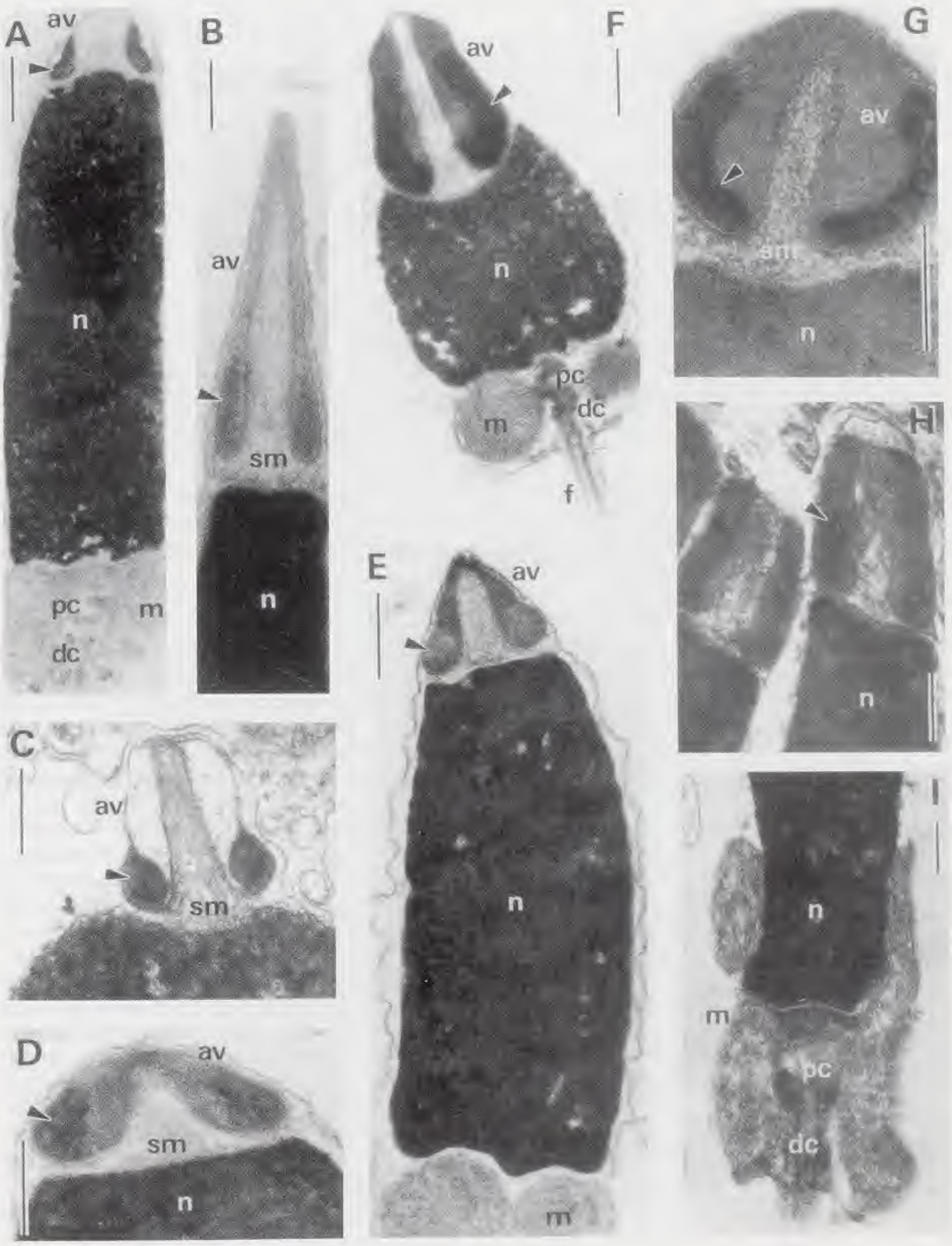
FIG. 2. — **A-E:** Electron micrographs illustrating major variations between Veneroida examined. Group A. **A:** Sperm head and midpiece of *Codakia punctata* (Lucinidae, Lucinoidea). **B:** Acrosome of *Glauconome* sp. (Glauconomidae, Veneroidea). Arrowhead in this and other figures indicates basal ring component of acrosomal vesicle contents. **C:** Acrosome of *Spisula trigonella* (Mactridae, Mactroidea). **D:** Acrosome of *Fragum unedo* (Cardiidae, Cardioidea). **E:** Sperm head and midpiece of *Circe* cf *plicatina* (Veneridae, Veneroidea). Note curved nucleus. **F:** Sperm head, midpiece and proximal portion of flagellum of *Donax deltoides* (Donacidae, Tellinoidea). **G:** Acrosome of *Trapezium sublaevigatum* (Trapeziidae, Arcticoidea). **H:** Acrosome of *Tellina rostrata* (Tellinidae, Tellinoidea). **I:** Nuclear base and midpiece of *T. rostrata*. Note extensive overlap of mitochondria and nucleus. av, acrosomal vesicle; dc, distal centriole; f, flagellum; m, mitochondria (of midpiece); n, nucleus; pc, proximal centriole; sm, subacrosomal material. Scale bars: A, E, F = 0.5 µm; B-D, G-I = 0.25 µm.

Source : MNHN, Paris



Source : MNHN, Paris

mitochondria oblong and overlapping significantly with base of nucleus (similar to *Tellina* from Group C).

This represents one of the most modified sperm morphologies encountered in the Veneroida. The type is of special interest because microtubules surround the spermatid nucleus during condensation (at least in *Scrobicularia*; details unavailable for *Corbicula*). Spermatid perinuclear microtubules occur in no other bivalves, but are widely reported in gastropods (introsperm-producing taxa [15, 25, 26]), cephalopods [16] and polyplacophorans [3]. The presence of this sperm type in the Tellinoidea and Corbiculoidea raises important questions concerning the relationship between these superfamilies (see Systematic considerations below). A report (based on SEM and light microscopy) that spermatozoa of *Corbicula fluminea* are both dimorphic (head region either slender or wide) and biflagellate [28], requires confirmation using transmission electron microscopy.

Group E: Carditoidea (Carditidae, Crassatellidae) [18]. (Fig. 1E)

Acrosomal vesicle elongate conical, almost totally invaginated, with the presumed homologue of the basal ring evident as a dense inner layer. Subacrosomal material differentiated into axial rod. Nucleus rod-shaped with short but distinct apical depression (partly accommodating subacrosomal material). Midpiece characterized by 8 (rarely 7 or 9) tightly adpressed mitochondria surrounding a dense centriolar rod (a metamorphosed proximal centriole) and distal centriole.

This is a highly distinctive sperm type, and its presence in the Carditidae and Crassatellidae has wide taxonomic and phylogenetic implications within the Veneroida (see below). The transformation of the proximal centriole into a rod connecting distal centriole (basal body) to nuclear fossa is unique among molluscan aquasperm.

Phylogenetic and systematic considerations

According to ALLEN [1] the success of veneroid bivalves has pivoted on their exploitation of soft sediments, largely as a consequence of their possessing and developing siphons (in combination, it should be stressed, with an ability to burrow effectively). Although most adaptive radiation within the Veneroida took place within the Mesozoic, some superfamilies such as the Lucinoidea and Crassatelloidea extend back to the lower Palaeozoic [1, 6, 31, 32] while several important living families and genera date only from the late Cretaceous or Tertiary [31]. Veneroid origins are unclear, and it remains uncertain as to whether the group is truly monophyletic or not.

In the present account, five principal sperm morphologies are recognized within the Veneroida based on shared ultrastructural features (observed through TEM). Light microscopic work of KARPEVICH [23] indicates that helical sperm nuclei also occur in several species of Cardiidae and at least one species of Tellinidae. Present knowledge of comparative sperm ultrastructure in the Veneroida, despite the absence of data for a number of families and genera, provides new information relevant to discussions of relationships within the order.

Group A. Despite anatomical specializations in some living representatives, the Lucinoidea constitute one of the oldest living heterodont superfamilies, being definitely recorded from the Silurian [1, 5] but possibly extending back to the Ordovician if a close relationship with the fossil genus *Babinka* is accepted [30, 31, 40]. The presence of Group A sperm morphology in this superfamily, and its widespread occurrence within the Veneroida, suggest that the earliest members of the order also possessed Group A type spermatozoa. Comparative spermatology in Group A superfamilies, especially in relation to acrosomal and nuclear features, appears to have considerable taxonomic potential at the species and generic levels (e.g. in the Veneroidea [10]; Cardioidea [*]; Solenoidea [21]). The disputed superfamily placement of the Hemidonacidae [45], either among the Cardioidea or the Tellinoidea, could probably be settled by examination of sperm ultrastructural features, although it should be added that within at least one tellinoidean family

Source : MNHN, Paris

(Donacidae) the acrosome may be of Group A type (*Donax trunculus* only - [47]) or Group C (all four other investigated species of *Donax*).

Group B. The Galeommatoidea (= Leptonacea) are small, often hermaphroditic veneroids which fertilize eggs within the mantle cavity (where young are brooded) and employ a range of methods to transfer sperm from individual to individual (spermatophores, sperm morulae, usage of dwarf males) [35-38]. In addition, the only confirmed cases of sperm dimorphism within the Bivalvia involve galeommatoidean species [35].

The classification of galeommatoidean bivalves (= Leptonacea) is far from being fully resolved [2, 8], and this seems to be reflected in marked acrosomal differences between examined genera. For example, in *Lasaea*, *Scintilla* and *Divariscintilla* (Group B), the acrosomal complex is apically compressed and tilted at a considerable angle. In addition the basal ring is developed transversely (Fig. 1B). By contrast, in *Mysella* (placed in Group C), the acrosomal vesicle tapers apically and is not tilted. The basal ring is developed longitudinally and accompanied by a lamellate apical density [35]. Unfortunately the only available TEM micrograph of *Pseudopythina* spermatozoa [37] is not detailed enough to determine the substructure of the acrosome. Although galeommatoideans probably arose through neoteny [1], the actual source (? or sources) of these bivalves has not been identified with any certainty. Available sperm data suggest that all galeommatoideans could have arisen from the Tellinoidea or the Arcticoidea, or possibly from both if the superfamily proves not to be monophyletic.

Group C. Spermatozoa of Group C could have been easily derived from those of Group A through lengthening and an increase in complexity of the basal ring component of the acrosomal vesicle. Within the Tellinoidea, spermatozoa of the Donacidae are typical of Group C, while those of *Tellina rostrata* (Tellinidae) appear to bridge the gap between Groups C and D by having significant mitochondrial overlap with the nucleus. It is unfortunate that so few tellinoidean families have been examined for sperm ultrastructure (e.g. no available data for the Solecurtidae, Semelidae, Psammobiidae). Such information would be of considerable value not only in evaluating relationships within the Tellinoidea, but also in exploring possible connections with the Arcticoidea, Galeommatoidea and freshwater Dreissenoidea. At the species level, comparative sperm morphology within the Donacidae may prove to be of considerable taxonomic use. Spermatozoa of the Australian species *Donax deltoides* are structurally similar to, although not identical with, those of the South African species *D. sordidus* and *D. madagascariensis* but differ markedly in acrosomal shape from another South African species *D. serra* (compare Fig. 1C, 2F with results by HODGSON *et al.* [20]). Spermatozoa of all four of these species of *Donax* differ from those of *D. trunculus*, which has a more elongate nucleus and an acrosome essentially of the Group A type [47].

Group D. Particularly interesting is the similarity between spermatozoa of the tellinoidean *Scrobicularia plana* (the only investigated member of the Scrobiculariidae) (Fig. 1D) and the freshwater Corbiculoidea. Is it possible that the Corbiculoidea have been derived from the Tellinoidea, or are the observed sperm similarities between *Scrobicularia* and *Corbicula* merely the result of convergence (that is, a similar fertilization biology)? In the absence of comprehensive data for a range of tellinoidean and corbiculoidean species this must remain an intriguing but presently insoluble problem. BOSS [2] considered that the Scrobiculariidae were perhaps unnecessarily split from the large tellinoidean family Semelidae. Further research on the spermatozoa of semelids and scrobiculariids (Tellinoidea) will undoubtedly throw further light onto this issue.

Group E. A close relationship between the Carditidae and Crassatellidae is clearly indicated by sperm morphology providing strong support for YONGE's suggestion [50, 51] that the Carditoidea and Crassatelloidea should be united into a single superfamily (for further discussion see HEALY [18]). Although the relationship of Group E (Carditidae + Crassatellidae) to other Veneroida is extremely uncertain, the preponderance of apomorphic sperm characters in this

Source : MNHN, Paris

Group effectively excludes it as a source of other veneroid taxa (e.g. origin of Veneroidea, Tellinoidea, Chamoidea plus Myoida from the "Carditida" suggested by SCARLATO & STAROBOGATOV [44]; origin of Cardioidea from carditids or crassatellids suggested by KEEN [24], origin of Tridacnidae from Carditoidea suggested by ALLEN [1]).

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Source : MNHN, Paris

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Source : MNHN, Paris

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Source : MNHN, Paris

Spermatozoal Morphology of Patellogastropoda and Vetigastropoda (Mollusca: Prosobranchia)

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ABSTRACT

The morphologies of the aquasperm of Patellogastropoda and Vetigastropoda are distinctly different, adding further support to the view that these two taxa are not closely related. Within the patellogastropods each family examined to date (Patellidae, Nacellidae, Lottiidae, Acmaeidae) has sperm with distinguishing features and it is therefore possible to recognize members of a family and differentiate between families using sperm morphology. Similarly sperm morphology can be used to differentiate between families of vetigastropods. Although there are a few exceptions, the size (length to breadth ratio) and shape of the nucleus and acrosome of sperm of species within each family are similar. The broad similarities in the morphology of the spermatozoa of the Pleurotomarioidea, Fissurelloidea, Haliotoidea and Trochoidea (with the exception of the Skeneidae) indicate that these taxa are phylogenetically closely related. Some vetigastropods, notably the Lepetodrilidae, Scissurellidae and Skeneidae, have modified spermatozoa which correlates to internal fertilization or fertilization within the mantle cavity. It is suggested that more extensive studies on the sperm from these families as well as other archaeogastropod taxa could provide additional clues to the phylogeny of higher gastropods.

RÉSUMÉ

La morphologie des spermatozoïdes des Patellogastropoda et des Vetigastropoda (Mollusca: Prosobranchia)

La morphologie des aquaspermatozoïdes est différente chez les Patellogastropoda et les Vetigastropoda, ce qui ajoute des arguments à l'opinion que ces taxons ne sont pas proches. Parmi les Patellogastropoda chacune des familles examinées jusqu'ici (Patellidae, Nacellidae, Lottiidae et Acmaeidae) a des spermatozoïdes avec des caractères distinctifs et il est donc possible de reconnaître les membres d'une famille et de différencier les familles en utilisant la morphologie du spermatozoïde. De même, la morphologie du spermatozoïde peut être utilisée pour différencier les familles de Vetigastropoda. En dépit de quelques exceptions, les dimensions (rapport longueur sur largeur) et la forme du noyau et de l'acrosome des spermatozoïde des espèces d'une même famille sont similaires. La grande ressemblance morphologique des spermatozoïdes des Pleurotomarioidea, Fissurelloidea, Haliotoidea et Trochoidea (à l'exception des Skeneidae) indique que ces taxons sont très proches phylogénétiquement. Quelques Vetigastropoda, en particulier les Lepetodrilidae, Scissurellidae et Skeneidae, ont des spermatozoïdes modifiés qui sont corrélés avec la fécondation interne ou la fécondation à l'intérieur de la cavité palléale. Il est probable que des études plus nombreuses des spermatozoïdes de ces familles et d'autres Archaeogastropodes pourraient fournir des indices supplémentaires pour la phylogénie des Gastéropodes évolués.

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Source : MNHN, Paris

Comparative spermatology has without doubt made significant contributions to a greater understanding of the taxonomic and phylogenetic relationships within and between many taxa. Within the molluscan class Gastropoda, the archaeogastropods are a large assemblage of prosobranchs, the taxonomic status of which has been the subject of considerable debate [5, 6, 9, 16]. Much of this debate is a result of the discovery of new taxa from deep-sea and hydrothermal vent communities [6-9, 31]. Thus in several recent re-evaluations of the Archaeogastropoda (*s. lat.*) [7-9, 16, 30], the assemblage has been split into numerous new orders.

A number of the recent publications have also attempted to reconstruct phylogenetic relationships within the archaeogastropods *(s. lat.)* as well as between archaeogastropods and higher gastropod taxa (e.g. caenogastropods and euthyneurans) [5-7, 16, 17]. To date most of these studies have not incorporated detailed information from sperm morphology (or spermiogenesis), information which may provide valuable insights into archaeogastropod taxonomy and phylogeny.

Two of the recognized orders within the archaeogastropod assemblage are the Patellogastropoda (formally Docoglossa) and Vetigastropoda. Whereas virtually nothing is known about sperm morphology of the recently discovered archaeogastropods, in the last 14 years there have been a number of publications on the structure of the spermatozoa of patellogastropods [1, 18-21, 25-28, 34] and vetigastropods [10, 13-15, 22, 23]. This paper reviews this information, discusses the systematic and phylogenetic implications of the data and highlights gaps in our knowledge.

MATERIALS AND METHODS

Information on the ultrastructure of the spermatozoa of Patellogastropoda and Vetigastropoda was obtained either from published papers (see Table 1 and reference list) or from tissue prepared for transmission electron microscopy. For TEM, small portions of the testis of each species were fixed in 2.5% glutaraldehyde in filtered sea water for 2-3 hours. The exception to this was the hydrothermal vent gastropod *Lepetodrilus fucensis*. This material spent several weeks in the glutaraldehyde fixative. Tissues were post-fixed in 1% osmium tetroxide in 0.1M sodium cacodylate buffer and filtered sea water for 90 minutes, dehydrated in a graded ethanol series, and embedded in a TAAB/Araldite resin mixture via propylene oxide. Thin sections (silver/gold) were stained in 5% aqueous uranyl acetate (30 minutes) and lead citrate (5 minutes) and examined in a JEOL JEM CXII TEM at 80kV.

RESULTS

Patellogastropoda

The spermatozoa of 43 species from four families (Table 2) of Patellogastropoda have been examined or described and all have aquasperm. Results to date suggest that members of each family of patellogastropod have spermatozoa with characteristic morphological features (Fig. 1).

Patellidae. In species of Patellidae (Fig. 1A-E), the acrosome, the contents of which may or may not be differentiated into electron-dense and electron-lucent regions, constitutes <50% of the head length and is deeply invaginated posteriorly. The subacrosomal space does not contain an axial rod. Despite these similarities within the Patellidae five morphological types of spermatozoa can be recognized within the family (Fig. 1A-E). The first type (Fig. 1A) have heads with cylindrical nuclei (usually <5 µm long and "bullet-shaped") which are rounded anteriorly and small cap-like acrosomes, the contents of which are uniformly electron-dense (i.e. undifferentiated) (11 species). The second type (Fig. 1B; 3 species) have very elongate "flask-shaped" nuclei (>12 µm long) which are pointed anteriorly and acrosomes with long anterior extensions. The third type (Fig. 1C; 11 species) have nuclei (about 5 µm long) which have a square-shaped anterior which intrudes into the subacrosomal space giving the nucleus a "bottle-shaped" appearance. Anterior to the nucleus is a relatively large acrosome, the wall of which is bulbous posteriorly and in addition the contents are differentiated into electron-dense and electron-lucent regions. The fourth type (Fig. 1D), have cylindrical nuclei (5-7 µm long) and an acrosome

Source : MNHN, Paris

TABLE 1. — Summary of the morphological features of the spermatozoa of vetigastropod families. * Information available as published diagram only. ** Deep-sea species.

Taxon	Reference	Common features of sperm
Pleurotomarioidea		
Pleurotomariidae		Head: Cylindrical (L:B=3:1)
Perotrochus westralis	[10]	Nucleus: Cylindrical (L:B, 2.5:1) with shallow anterior fossa.
Perotrochus quoyanus	[15]	Acrosome: Broadly conical, <20% head length, undifferentiated.
Haliotoidea		
Haliotidae		Head: Cylindrical (L:B>5:1).
Haliotis discus	[33]	Nucleus: Narrow anterior fossa.
Haliotis rufescens	[29]	Acrosome: Comprises 40-50% head length, with posterior
Haliotis midae	[22]	invagination in form of narrow canal with axial rod.
Haliotis diversicolor aquatilis	[28]	
Fissurelloidea		
Fissurellidae		Head: Cylindrical (L:B>4:1).
Fissurella mutabilis	[22]	Nucleus: Cylindrical (L:B>3:1), Eumarginulinae with deep U-
Fissurella natalensis	[22]	shaped anterior fossa, Fissurellinae fossa absent but small
Dendrofissurella scutellum	[22]	anterior extension present.
Amblychelipas javanicens	[14]	Acrosome: usually 20-30% of head length, with deep posterior
Monifortula conoidea	[14]	invagination. Axial rod present in Eumarginulinae.
*Scutus antipodes**	[14]	
Scutus unguis	[22]	
*Scutus sinensis**	[28]	
*Macroschisma sinense**	[28]	
Diodora aspera	[21]	
Scissurelloidea		
Scissurellidae		Modified spermatozoa (Ent-aquasperm?).
Sinezona sp.	[14]	Nucleus: Deep anterior and posterior fossae
		Acrosome: Undifferentiated, very small (13% head length), cap-like.
Trochoidea		
Trochidae		Head: Short cylindrical (L:B <3:1).
Oxystele variegata	[24]	Nucleus: Usually short (L:B >2:1); U-shaped anterior fossa.
Oxystele impervia	[24]	Acrosome: Comprises 50% or less of head length, usually
Oxystele tabularis	[24]	undifferentiated internally. Axial rod present.
Oxystele sinensis	[24]	
Oxystele tigrina	[24]	
Gibbula umbilicalis	[2, 3]	
Gibbula tumida	[27]	
Monodonta turbinata	[27, 18]	
Monodonta australis	[22]	
Monodonta labio labio	[28]	
Trochus nigropunctatus	[22]	
*Chlorostoma argyrostoma lischkei**	[28]	
Omphalus pfeifferi	[28]	
Tectus pyramis	[28]	
Austrocochlea obtusa	[11]	
*Calliotropis glyptus***	[12]	
Turbinidae		
Turbo coronatus	[22]	Head: Broad cylinder (L:B >3:1).
Turbo sarmaticus	[22]	Nucleus: L:B <1.5:1; Very wide V-shaped anterior fossa.
Turbo cidaris natalensis	[22]	Acrosome: Comprises >50% of head length, differentiated
Lunella cinerea	[28]	internally with short narrow posterior invagination.
Lunella granulata	[28]	
*Batillus cornutus**	[28]	
Phasianellidae		
Tricolia capensis	[22]	Head: Short cylinder.
		Nucleus: Short and barrel shaped with U-shaped anterior fossa.
		Acrosome: n-shaped, <30% head length, undifferentiated internally.
Skeneidae		Dimorphic and modified sperm - see Fig. 2.
Zalipais laseroni	[13]	
Microcarina surgerea	[13]	
Liotella parvirota	[13]	
Lepetodriloidea		
Lepetodrilidae		Modified sperm (Ent-aquasperm ?) - see Fig. 2.
Lepetodrilus fucensis	[23]	Nucleus: Elongate cylindrical and rounded anteriorly.
		Acrosome: Modified subacrosomal plate.

Source : MNHN, Paris

TABLE 2. — The species of four families of patellogastropod for which descriptions of the sperm exist. Information obtained from [1, 18-21, 25, 28, 35]. The sperm types of Patellidae (I, II, III, IV, VI) are from [19, 25]. [1]Indicates species of uncertain taxonomic status but initially identified as *P. miniata* from Angola; [2]Indicates species of uncertain taxonomic status but initially identified as *P. miniata* from Namibia.

PATELLIDAE	*Patella aspera*
Sperm Type I:	*Patella rustica*
Patella aphanes	*Patella depressa*
Patella obtecta	*Patella ferruginea*
Patella barbara	*Patella caerulea*
Patella argenvillei	*Patella candei*
Patella tabularis	**Sperm Type IV:**
Patella chapmani	*Helcion pectunculus*
Patella laticostata	*Helcion pruinosus*
Patella flexuosa	**Sperm Type III:**
Patella cochlear	*Helcion dunkeri*
Patella longicosta	*Helcion pellucidus*
Sperm Type II:	
Patella granularis	NACELLIDAE
Patella miliaris	*Nacella delesserti*
Patella peronii	*Cellana capensis*
Sperm Type III:	*Cellana testudinaria*
Patella granatina	*Cellana toreuma*
Patella oculus	
Patella concolor	LOTTIIDAE
Patella cf. *miniata*[1]	*Lottia pelta*
Patella safiana	*Lottia digitalis*
Patella canescens	*Lottia strigatella*
Patella compressa	*Tectura scutum*
Patella miniata	
Patella cf. *miniata*[2]	ACMAEIDAE
Sperm Type VI:	*Patelloida profunda albonontata*
Patella vulgata	*Patelloida saccharina lanx*

which is differentiated internally and has a cylindrical posterior lobe. Finally the fifth type (Fig. 1E; 7 species) consists of a nucleus which is elongate and cylindrical but capped by a short, undifferentiated A-shaped, conical acrosome.

Nacellidae. In the Nacellidae the heads of the spermatozoa are always elongate (length: breadth >7:1) and the nucleus is distinctly conical in shape (Fig. 1F). The acrosome, which constitutes <50% of the head length, has a complex internal differentiation which can take the form of electron-opaque striations (e.g. *Cellana capensis* [19]). An axial rod is always present in the subacrosomal space.

Lottiidae. Lottiidae spermatozoa have a head with a short cylindrical nucleus which is rounded anteriorly (Fig. 1G). Anterior to the nucleus is an elongate acrosome which constitutes >50% of the total head length. The acrosome is invaginated posteriorly, differentiated internally into an outer electron-lucent and inner electron-dense region and has an elongate posterior lobe which protrudes into the sub-acrosomal space. Within this space, the fibrous material is aggregated into an axial rod-like structure. The midpiece has four to five spherical mitochondria (with well developed cristae), which surround the proximal and distal centrioles. Surrounding the anterior portion of the axoneme is an elongate (1 µm) cytoplasmic collar (this structure is much smaller in other patellogastropod taxa).

Source : MNHN, Paris

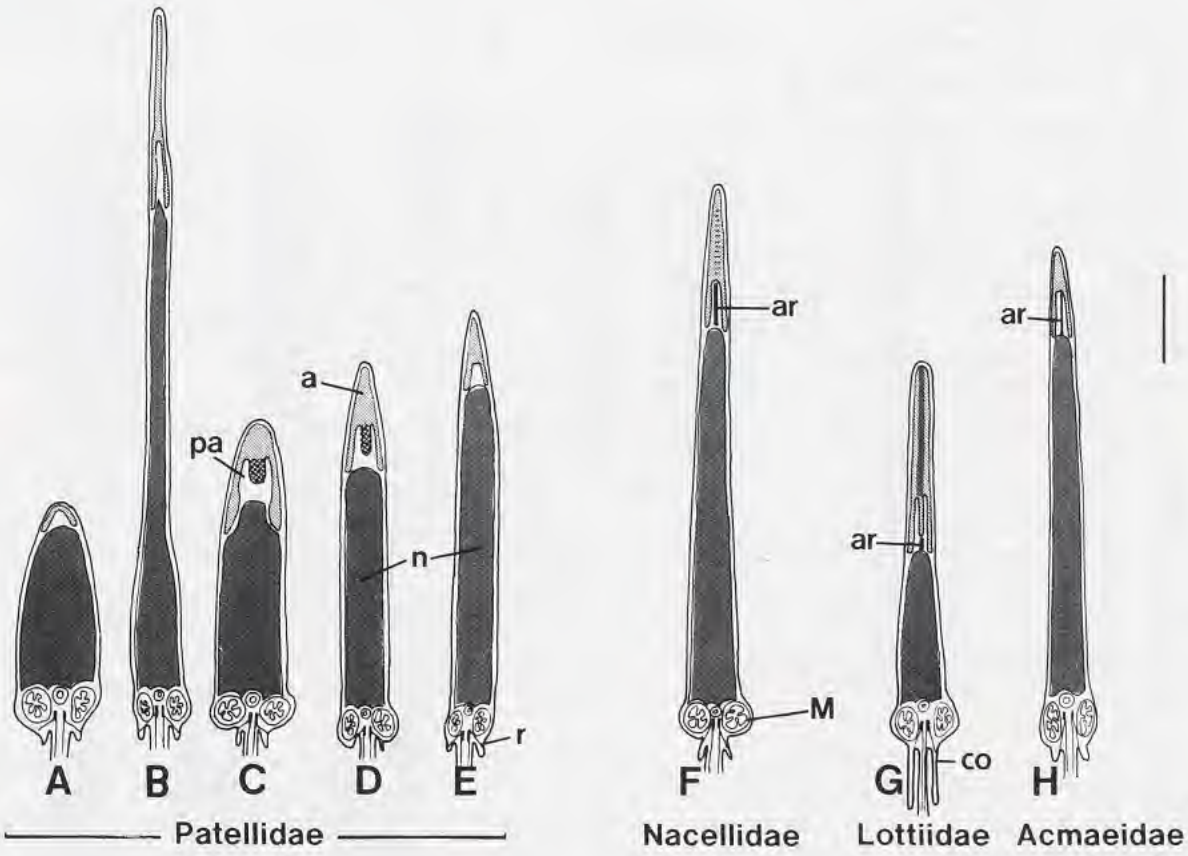

FIG. 1. — Semi-diagrammatic longitudinal sections through the spermatozoa of representative Patellogastropoda. a, acrosome; ar, axial rod; co, cytoplasmic collar; M, mitochondrion; n, nucleus; pa, posterior acrosomal invagination. Diagrams A-C, F, G are modified from [21]. Scale bar = 2 µm.

Acmaeidae. Only two species, *Patelloida profunda albonotata* [18] and *P. saccharina lanx* [28], have been described from this family, the sperm of the latter species being illustrated diagrammatically only. The sperm have elongate, cylindrical heads (about 5.6 µm long x 0.3 µm mid-diameter in *P. p. albonotata* and 5.4 µm x 0.4 µm in *P. s. lanx*) (Fig. 1H). The nucleus is cylindrical, tapering towards the rounded anterior, and is capped by a short conical acrosome (about 1 µm long) the contents of which are undifferentiated. The acrosome has a broad posterior invagination, the subacrosomal space containing an axial rod. The midpiece contains four to five spherical mitochondria (about 0.5 µm diameter).

Vetigastropoda

With the exception of the Skeneidae which have dimorphic spermatozoa [13], vetigastropods produce only euspermatozoa. Although the majority of species have aquasperm (Fig. 2) each family has spermatozoa with characteristic features (summarized in Table 2).

Pleurotomariidae. Only two species from this family, *Perotrochus westralis* (described as *Pleurotomaria africana*) and *Perotrochus quoyanus* have been examined [10, 15]. Both species have aquasperm. The sperm head is composed of a cylindrical nucleus (length: breadth about 3:1)

Source : MNHN, Paris

which is square anteriorly with a small central invagination. Posteriorly the nucleus has a small but well developed fossa (described as crypt-like by Healy & Harasewych [15]).

Fibrous rootlets extend from the proximal centriole into this fossa. The acrosome is in the form of a rounded cone the contents of which are not differentiated. In addition the acrosome has a deep, narrow posterior invagination.

Haliotidae. The sperm of the Haliotidae all have a head which is cylindrical, with a L:B ratio normally >5:1 (Table 1). The nucleus, which is cylindrical, has a narrow anterior fossa. The acrosome, which constitutes ~40-50% of the total length of the sperm head has a narrow anterior canal containing an axial rod. The acrosomal contents may or may not be differentiated.

Scissurellidae. The spermatozoon of only one species of scissurellid, *Sinezona* sp. has been described [14] and it is of the ent-aquasperm type [32]. The head consists of a cylindrical nucleus which has prominent anterior and posterior invaginations. The anterior invagination houses an axial rod. The small cap-shaped acrosome is undifferentiated internally. The midpiece consists of an axoneme which is surrounded by a sleeve-like mitochondrion.

Fissurellidae. Most fissurellids have a sperm head with a length to breadth ratio of >4:1. The nucleus is cylindrical (L:B 3:1; *Montfortula conoidea* and *Scutus antipodes* are exceptions [14]). Except for *M. conoidea* and *S. antipodes* in which the acrosome comprises 50% of the head length, the acrosome of fissurellids is nearly always small, <35% of the total head length and is deeply invaginated, the invagination penetrating the acrosome as a narrow tube which widens at the anterior. In the subfamily Fissurellinae the nucleus either has a small peg-like anterior extension (includes species of *Fissurella* and *Dendrofissurella*) or there is a shallow anterior nuclear fossa (*Amblychilepas*). By contrast the Eumarginulinae (includes species of *Montfortula* and *Scutus*) all have a large V-shaped anterior nuclear invagination which houses an axial rod.

Trochidae and Phasianellidae. The sperm of species from these two families have a barrel-shaped nucleus (L:B <4:1) which has a U-shaped anterior invagination. In many species the contents of the broadly conical acrosome are uniformly electron-opaque [22], whereas in other species a differentiated acrosome has been noted [10]. The acrosome normally constitutes 50% or less of the total head length and has a narrow posterior invagination. In most trochids there is an axial rod.

Turbinidae. Turbinid spermatozoa are characterised by a nucleus with a length to breadth ratio <1.5:1. The nucleus has a very wide anterior invagination and a relatively large (>50% of the total head length) conical acrosome, the base of which lies within the nuclear invagination. The acrosomal contents are differentiated internally. Posteriorly the acrosome has a short, narrow invagination.

Lepetodrilidae. A preliminary examination of one species (*Lepetodrilus fucensis*) from this taxon, has revealed that they produce only euspermatozoa which are modified [23]. The sperm have an elongate head (about 11 µm long) comprised of a long cylindrical nucleus (about 9 µm long x 0.4 µm mid-diameter) and an anteriorly positioned conical acrosome (about 2 µm long). Between the acrosome and the nucleus is a tube-like structure, the subacrosomal plate (about 0.3 µm long), which is expanded inwards at its base as a small flange. The lumen of this tube is filled with an amorphous material. The acrosome, which sits on the tube, is deeply invaginated posteriorly; the subacrosomal space within the invagination contains an axial rod.

Posteriorly the nucleus has a shallow invagination into which the complex centriolar apparatus protrudes. A single large mitochondrion it sited laterally to the centriolar apparatus. The anterior portion of the axoneme (which emerges from the centriolar complex) is surrounded by a cytoplasmic collar which forms a tube about 2.5 µm long. The cytoplasmic collar contains numerous tubular structures (about 60 nm diameter) which encircle the axoneme.

Source : MNHN, Paris

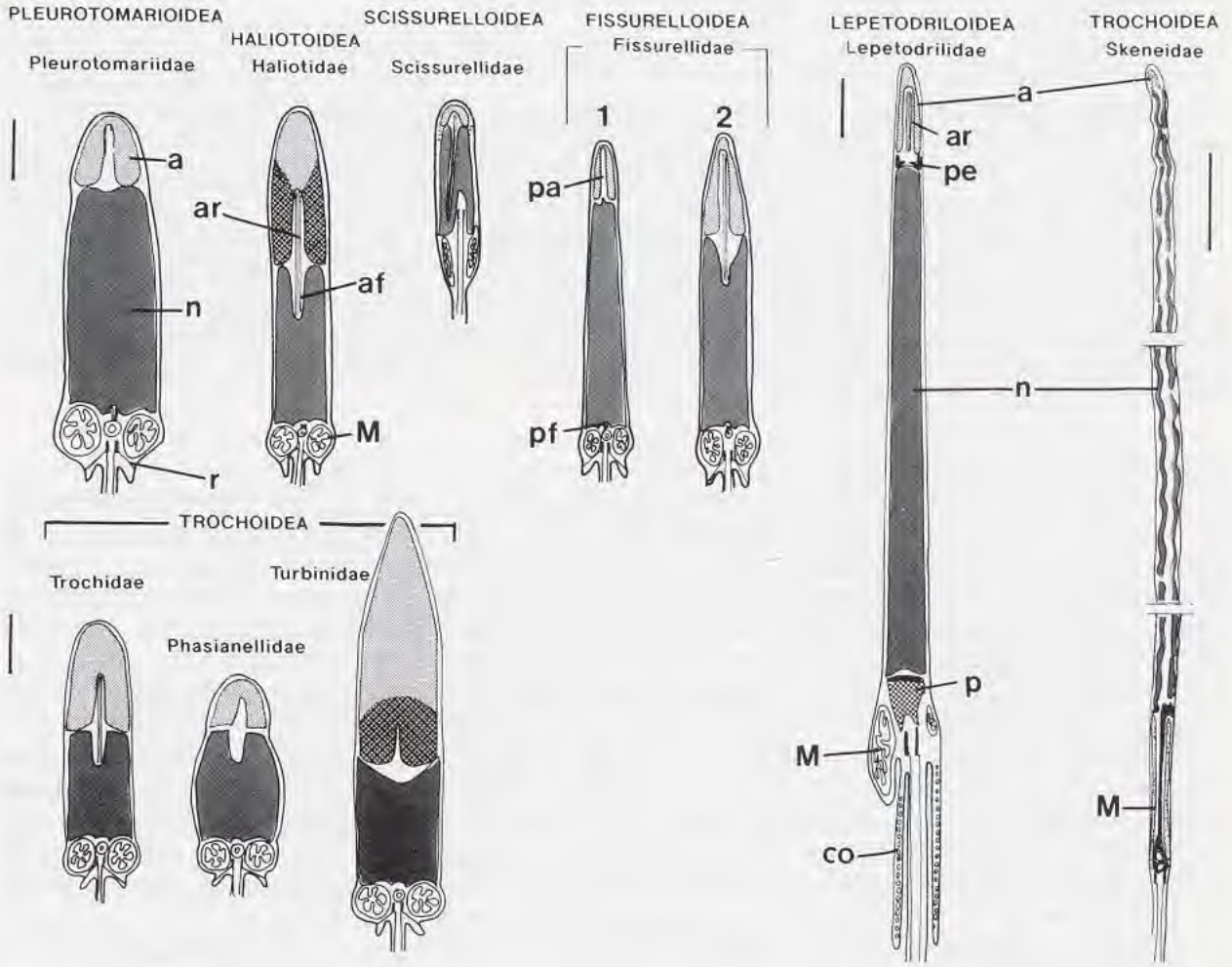

FIG. 2. — Semi-diagrammatic longitudinal sections through spermatozoa of nine families of Vetigastropoda. Examples chosen are thought to typify each family. a, acrosome; af, anterior nuclear fossa; ar, axial rod; co, cytoplasmic collar; M, mitochondrion; n, nucleus; p, proximal centriole; pa, posterior acrosomal invagination; pe, subacrosomal plate; pf, posterior nuclear fossa, r, radial arm. Within the Fissurellidae type 1 sperm are from the Fissurellinae and type 2 from the Eumarginulinae. The diagrams are modified from [13, 22, 23]. Scale bars = 1 µm.

Skeneidae. The sperm of three species of skeneid have been described [13]. All produce euspermatozoa and one species (*Zalipais laseroni*) has paraspermatozoa. The eusperm are uniflagellate with a long (about 50 µm in *Zalipais laseroni*), tubular helically coiled nucleus. Anterior to the nucleus of *Z. laseroni* there is a small conical acrosome. The midpiece consists of a mitochondrial sleeve which surrounds an electron-dense rod (about 3 µm long).

The parasperm of *Z. laseroni* consist of an elongate electron-dense head, a short midpiece of centriolar rods and mitochondria, and a posterior tuft of flagella.

Source : MNHN, Paris

DISCUSSION

Patellogastropod-Vetigastropod relationships

LINDBERG [30] has argued that limpets from the superfamilies Patelloidea, Nacelloidea and Acmaeoidea have morphological features which differ from those of other archaeogastropods. These limpets were therefore removed to a separate order, Patellogastropoda and are regarded as an early holophyletic gastropod offshoot [7, 9]. It is now clear that there are also distinct differences in sperm morphology between the patellogastropods and vetigastropods. Patellogastropods always produce aquasperm, the nuclei of which never have an anterior fossa, whereas in the majority of vetigastropods with aquasperm (the Fissurellinae is an exception) this nuclear feature is present. Furthermore, in patellogastropods the acrosome always has a wide posterior invagination. By contrast in vetigastropods the posterior acrosomal invagination is always in the form of a narrow canal. These small but consistent differences in sperm morphology between patellogastropods and vetigastropods supports the view [7, 30] that these two taxa are distinct from each other.

Patellogastropod relationships

Although information on sperm morphology from families of Patellogastropoda is still incomplete, results to date suggest that each family has a sperm with distinguishing features. As the evolutionary trends and relationships between and within patellogastropods are not certain [30], spermatozoon morphology may eventually provide additional clues to limpet phylogeny when data from outstanding taxa (e.g. Lepetidae) and more detailed information from other taxa (e.g. Nacellidae and Acmaeidae) are forthcoming. Despite the incomplete data base, the information available on sperm morphology invites some speculation on limpet phylogeny. It is suggested that spermatozoa with the most plesiomorphic features are found in the Patellidae, with some species having a sperm with a short "bullet-shaped" nucleus and small undifferentiated acrosome (Fig. 1A). Apomorphic features of sperm of other species of Patellidae, as well as the Nacellidae, Lottiidae and Acmaeidae would therefore include a lengthening of the nucleus (often accompanied by an elaboration of the shape) and an increase in the size and complexity of the acrosome (Fig. 1B-G). Sperm morphology therefore adds further support to the view that within the Patellogastropoda, the patellids are the most primitive taxon [30].

From the few species described to date [18, 21, 28] (Table 2) the spermatozoa of the Lottiidae and Acmaeidae appear to be very similar. It is proposed that of the two taxa, the sperm of the Acmaeidae are more plesiomorphic, having smaller acrosomes which are slightly simpler in morphology (e.g. undifferentiated internally). As the fossil history for *Patelloida* (Acmaeidae) is greater than that of the Lottiidae [30], it might be expected therefore that the acmaeids would have more plesiomorphic sperm.

The morphology of the spermatozoa of most species of the Patellidae is now known [1, 18-21, 25, 26, 34] and the data have not only provided the first insights into relationships within this family of limpets but have also provided clues to their evolutionary radiation. Most species have one of three unequivocal types of sperm (designated Types I, III and VI by HODGSON *et al.* [25]; Table 2) and it is possible that they represent three lines of patellid radiation which have occurred in separate biogeographic areas. HODGSON *et al.* [25] suggest that species with type I sperm (Fig. 1A) probably had their centre of radiation in the Indo-Pacific, those with type III sperm (Fig. 1C) in the South-East Atlantic and finally those with type VI sperm (Fig. 1E) in the North East Atlantic and Mediterranean.

Vetigastropod relationships

The Vetigastropoda comprise the Pleurotomarioidea, Fissurelloidea, Haliotoidea, Trochoidea, Scissurelloidea and Lepetodriloidea. The broad similarities in the structure of the spermatozoa of the Pleurotomarioidea, Fissurelloidea, Haliotoidea, and Trochoidea add further

Source : MNHN, Paris

support to the contention [7, 10, 22, 24, 28] that these superfamilies are phylogenetically closely related. In particular, the spermatozoa of the pleurotomarids and the those of the Trochoidea (particularly the Trochidae) are very similar [10, 15] suggesting that these two superfamilies have a close relationship. Thus sperm morphology supports similar conclusions based on other morphological features [7, 8, 15].

It is suggested that within the Trochoidea, the most plesiomorphic sperm are found within the Trochidae and Phasianellidae whereas the spermatozoa of the Turbinidae have more apomorphic features. These features include a very wide anterior nuclear invagination and a conical acrosome (differentiated internally), the base of which lies within the nuclear invagination, which has lengthened, constituting >50% of the total head length. Sperm morphology therefore does not accord with other morphological features of the Turbinidae, the turbinids having retained many primitive pleurotomariacean features [17]. However it should be noted that HICKMAN & MCLEAN [17] regard the phasianellids as a subfamily of the Turbinidae. Nevertheless spermatologically the phasianellids are more similar to the trochids than to other turbinids (Fig. 2).

The Skeneidae are unlike other trochoideans in that the species examined to date (*Z. laseroni*) [13] has dimorphic spermatozoa, and in addition the euspermatozoa are of the modified (after FRANZÉN [4]) or introsperm (after ROUSE & JAMIESON [32]) type. Such spermatozoa are found in skeneid gastropods with internal fertilization and it is highly likely that skeneids have internal fertilization. The fact that skeneids have introsperm makes it difficult to use sperm morphology to assess their phylogenetic relationship to most other vetigastropods. However the similarity in the structure of the spermatozoa of the skeneids and some caenogastropods (notably the Cerithioidea) has led HEALY [13] to suggest that despite some morphological incongruities, the vetigastropods are the probable ancestral source of the caenogastropods.

Representative species from two other vetigastropod superfamilies have now been found to have "modified" spermatozoa, the Scissurellidae [14] and Lepetodrilidae [23]. In both cases modifications to sperm morphology can be linked to a modification in the biology of fertilization. The scissurellids do not have organs to facilitate internal fertilization and it has therefore been suggested that fertilization occurs in the mantle cavity [8]. The morphology of the sperm of both the Scissurellidae and Lepetodrilidae are consistent with an ent-aquasperm form (after ROUSE & JAMIESON [32]) supporting the idea of fertilization in the mantle cavity.

Studies on spermatozoon morphology of the Patellogastropoda and Vetigastropoda have provided interesting insights into relationships within and between these taxa. However if information from sperm morphology is to be incorporated into phylogenetic and cladistic studies, descriptions from outstanding taxa are required. Although all patellogastropods and most vetigastropods produce aquasperm which are essentially plesiomorphic, these sperm can yield apomorphic characters [26] making it possible to incorporate characters from sperm into cladistic analyses. Within the Patellogastropoda comprehensive data on the morphology of the sperm of the Patellidae exist, but far less is known about other families. Within the vetigastropods an examination of the sperm of hydrothermal vent and deep sea taxa, such as the Skeneidae may help in furthering an understanding of the evolution of higher gastropods. Finally, urgent attention needs to be given to other archaeogastropod taxa such as the Neolepetopsidae, Cocculiniformia, Peltospiroidea, Neomphaloidea, Seguenzioidea, Cyclophoroidea and Ampullarioidea. An examination of sperm from taxa such as these could contribute to a greater understanding of gastropod phylogeny.

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Source : MNHN, Paris

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Source : MNHN, Paris

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Source : MNHN, Paris

Source : MNHN, Paris

Comparative Silver Staining of Molluscan Spermatozoa

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ABSTRACT

Mollusc spermatozoa were studied by transmission electron microscopy with the application of the silver nitrate method. Silver stained some components of the acrosomal vesicle and of the cytoskeletal elements of these cells in a pattern specific for each species. The importance of this staining method in relation to phylogenetic relationships is presented and discussed.

RÉSUMÉ

Coloration comparée à l'argent des spermatozoïdes de Mollusques

Des spermatozoïdes de Mollusques ont été étudiés en microscopie électronique à transmission grâce à la méthode au nitrate d'argent. Dans chaque espèce, l'argent colore certains composants de la vésicule acrosomienne et les éléments cytoplasmiques de ces cellules d'une manière spécifique. L'importance de cette méthode de coloration pour les relations phylogéniques est présentée et discutée.

The ultrastructural characteristics of mature spermatozoa have been used to examine phylogenetic relationships and have also been related to the different aspects of reproductive biology of the organism [18, 38, 39, 50, 51, 54]. In this respect, a great deal of work has been done on the ultrastructure of spermatozoa in the Patellogastropoda and Archaeogastropoda (Prosobranchia) as well as in the Heterodonta and Pteriomorphia (Bivalvia) [1-3, 6-37, 39-53, 55, 56, 59, 61-66].

In recent years, we have attempted to show that the silver staining method can be a useful and easy way to better understand specific aspects of spermatogenesis [57, 59, 60], as well as for comparative phylogenetical studies [58, 61-64]. In the present study, we present and critically review the silver staining characteristics of mature molluscan spermatozoa, with emphasis on its importance as a tool for studying phylogenetic relationships. We also provide the detailed technique so as to enable other laboratories to reproduce this staining method.

SOUSA, M., OLIVEIRA, E., CARVALHEIRO, J., & OLIVEIRA, V., 1995. — Comparative silver staining of molluscan spermatozoa. *In*: JAMIESON, B. G. M., AUSIO, J., & JUSTINE, J.-L. (eds), Advances in Spermatozoal Phylogeny and Taxonomy. *Mém. Mus. natn. Hist. nat.*, **166** : 179-187. Paris ISBN : 2-85653-225-X.

Source : MNHN, Paris

MATERIALS AND METHODS

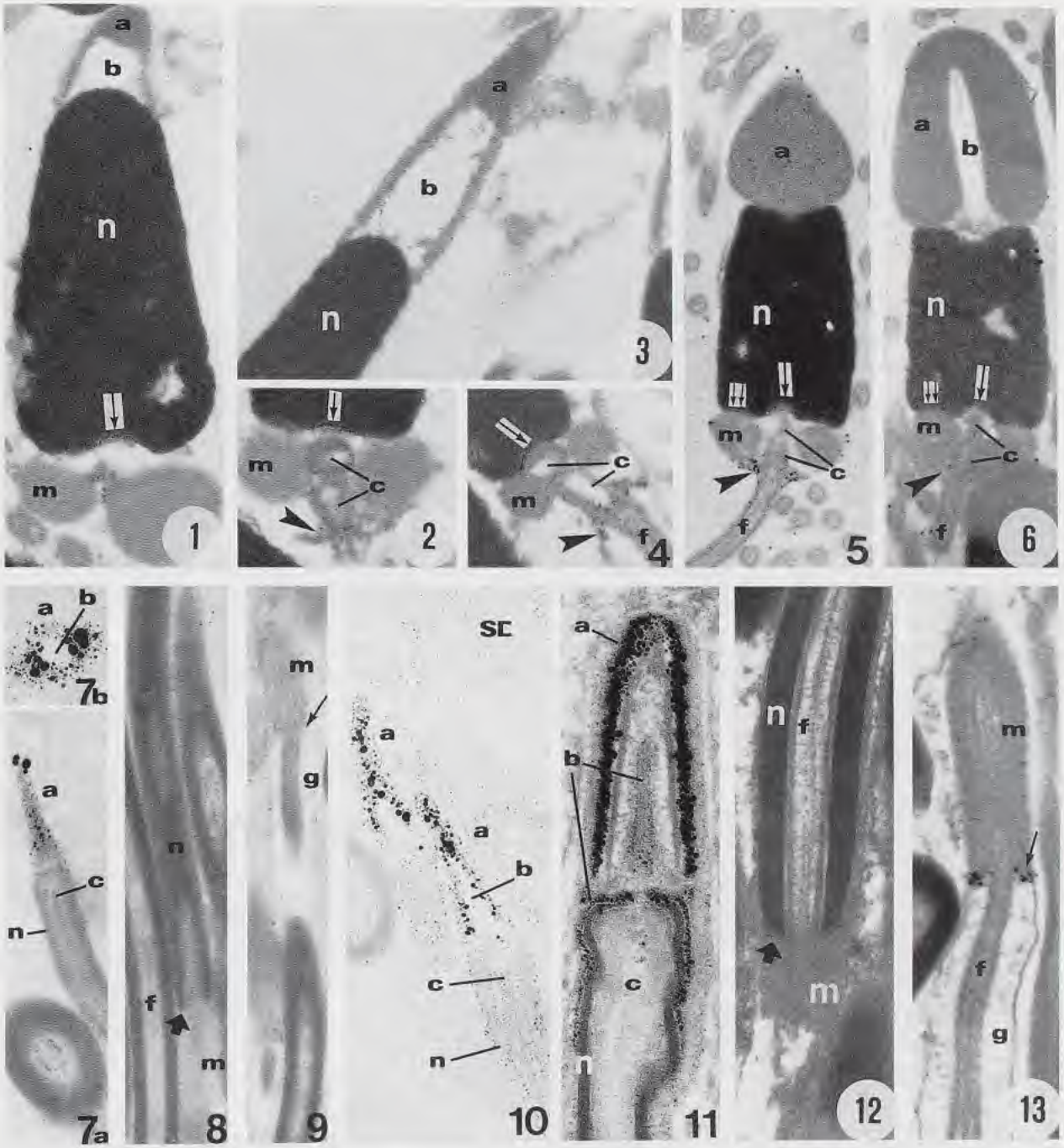
Specimens (Table 1) were collected from sands at the Atlantic coast of Portugal and Spain. Small pieces of testis (< 1 mm) were fixed with 2.5% glutaraldehyde buffered with 0.2 M Na Cacodylate, pH 7.4, for 2 h at 4°C, rinsed in the same buffer for 2 h at 4°C, postfixed in Carnoy (acetic acid:methanol, 1:3) for 5 min at room temperature, gradually rehydrated in ethanol (10 min in each step), and rinsed in distilled water for 30 min. They were then immersed in 50% aqueous silver nitrate (filtered by 0.2 µm) and placed in a water-bath under a Philips lamp (500 W) in such a way as to reach 55°C for 10 min. Finally, the specimens were rinsed in distilled water (3 x 2 min), incubated in an ammoniacal silver-formalin solution (2 g silver nitrate in 2.5 ml distilled water + 2.5 ml 33% ammonia solution + 5 ml 3% aqueous formalin, 0.2 µm filtered) for 3 min, rinsed in distilled water (3 x 2 min), dehydrated in an ethanol graded series, embedded in Epon, and examined by transmission electron microscopy.

TABLE 1. — Silver staining of mature spermatozoa in the Mollusca.
AV, acrosomal vesicle; SAM, subacrosomal material; CF/MF, centriolar fossa/mitochondrial fossa; PCS, material that links the proximal centriole to the nuclear base; Ce, centrioles; PCC/An, pericentriolar complex/annulus.

Taxa	AV	SAM	CF/MF	PCS	Ce	PCC/An	Reference
Gastropoda							
Patellogastropoda							
Helcion pellucidus	-	-	+	-	±	±	[63]
Patella rustica	-	-	+	-	±	+	[63]
Archaeogastropoda							
Gibbula umbilicalis	+	-	+	-	±	+	present study
Gibbula cineraria	-	-	±	-	±	±	present study
Caenogastropoda							
Littorina saxatilis	+	-	-	-	-	-	[58]
Littorina littorea	+	-	-	-	+	-	[58]
Nucella lapillus	+	+	+	-	-	+	[4, 5]
Bivalvia							
Heterodonta							
Veneroida							
Donax trunculus	+	-	+	+	+	+	[61]
Spisula solidissima	+	+	+	-	±	+	[61]
Cerastoderma edule	+	-	+	+	-	-	[58]
Scrobicularia plana	-	-	+	-	-	+	[61]
Pteriomorphia							
Mytiloidea							
Mytilus edulis	+	-	-	-	-	-	[58]
Ostreoidea							
Ostrea edulis	+	-	+	+	-	-	[62]
Crassostrea angulata	+	+	+	+	+	+	[62]

FIGS 1-13. — Prosobranchia. a, acrosomal vesicle; b, subacrosomal material; n, nucleus; m, mitochondria; c, centrioles; f, flagellum; g, glycogen sheath. **1, 2:** *Helcion pellucidus*. Silver stains the centriolar fossa (arrow), and lightly stains the centrioles (c) and the pericentriolar complex (arrowhead). x 29 000. **3, 4:** *Patella rustica*. Silver stains the centriolar fossa (arrow) and the pericentriolar complex (arrowhead), and lightly stains the centrioles (c). x 24 600. **5:** *Gibbula umbilicalis*. Silver stains the acrosomal vesicle (a), the centriolar fossa (arrow), the mitochondrial fossa (double arrow) and the pericentriolar complex (arrowhead), and lightly stains the centrioles (c). x 17 900. **6:** *Gibbula cineraria*. Silver lightly stains the centriolar fossa (arrow), the mitochondrial fossa (double arrow), the centrioles (c), and the pericentriolar complex (arrowhead). x 17 900. **7-9:** *Littorina saxatilis*.

Source : MNHN, Paris



Silver stains the acrosomal vesicle (a). Large arrow, mitochondrial fossa; small arrow, annulus. x 32 000; x 18 600; x 18 600. **10:** *Littorina littorea*. Silver stains the acrosomal vesicle (a) and the centriole (c). Results for the mitochondrial fossa and the annulus are as for *L. saxatilis*. SC, sertoli cell. x 41 000. **11-13:** *Nucella lapillus*. Silver stains the acrosomal vesicle (a), the subacrosomal material (b), the mitochondrial fossa (large arrow), and the annulus (small arrow). x 51 600; x 30 000; x 33 000.

Source : MNHN, Paris

RESULTS AND DISCUSSION

Silver staining has been used to demonstrate the argyrophilia of acidic phosphoproteins contained in nucleolar components and their metaphase counterparts [60]. However, silver staining has also been shown to stain, in invertebrate species, other nuclear and cytoplasmic structures of germ cells and thereafter used to show different staining characteristics in different sperm species [57-64].

In the present work, we update all results obtained in the Mollusca with this silver staining technique, and discuss its relative importance as a phylogenetical sperm marker (Table 1). In the Patellogastropoda, *Helcion pellucidus* and *Patella rustica*, which are morphologically different, exhibit a similar pattern of silver staining (Figs 1-4); in the Archaeogastropoda, *Gibbula umbilicalis* and *G. cineraria* differ in nuclear and acrosomal vesicle dimensions as also in their silver staining characteristics (Figs 5, 6); in the Caenogastropoda, despite the morphological similarity between *Littorina saxatilis*, *L. littorea* and *Nucella lapillus*, their silver staining characteristics are very dissimilar (Figs 7-13). In the Veneroida, *Donax trunculus*, *Spisula solidissima*, *Cerastoderma edulis* and *Scrobicularia plana* are all very different in their morphological aspects, and also present very distinctive silver staining characteristics (Figs 14-22). In the Mytiloidea (Pteriomorphia), only *Mytilus edulis* has been studied by silver staining (Figs 23, 24); but in the Ostreoidea, despite the morphological similarity between *Ostrea edulis* and *Crassostrea angulata*, both species can be easily and completely differentiated by silver staining (Figs 25-27).

In conclusion, the silver staining of mature spermatozoa can enable us to distinguish different sperm species which present identical ultrastructural characteristics, and it can also help in phylogenetical studies since spermatozoa of the same group frequently share several silver staining characteristics (Table 1). However, the validation of this cytochemical marker as a useful tool in phylogenetical studies awaits further studies on many other molluscan species.

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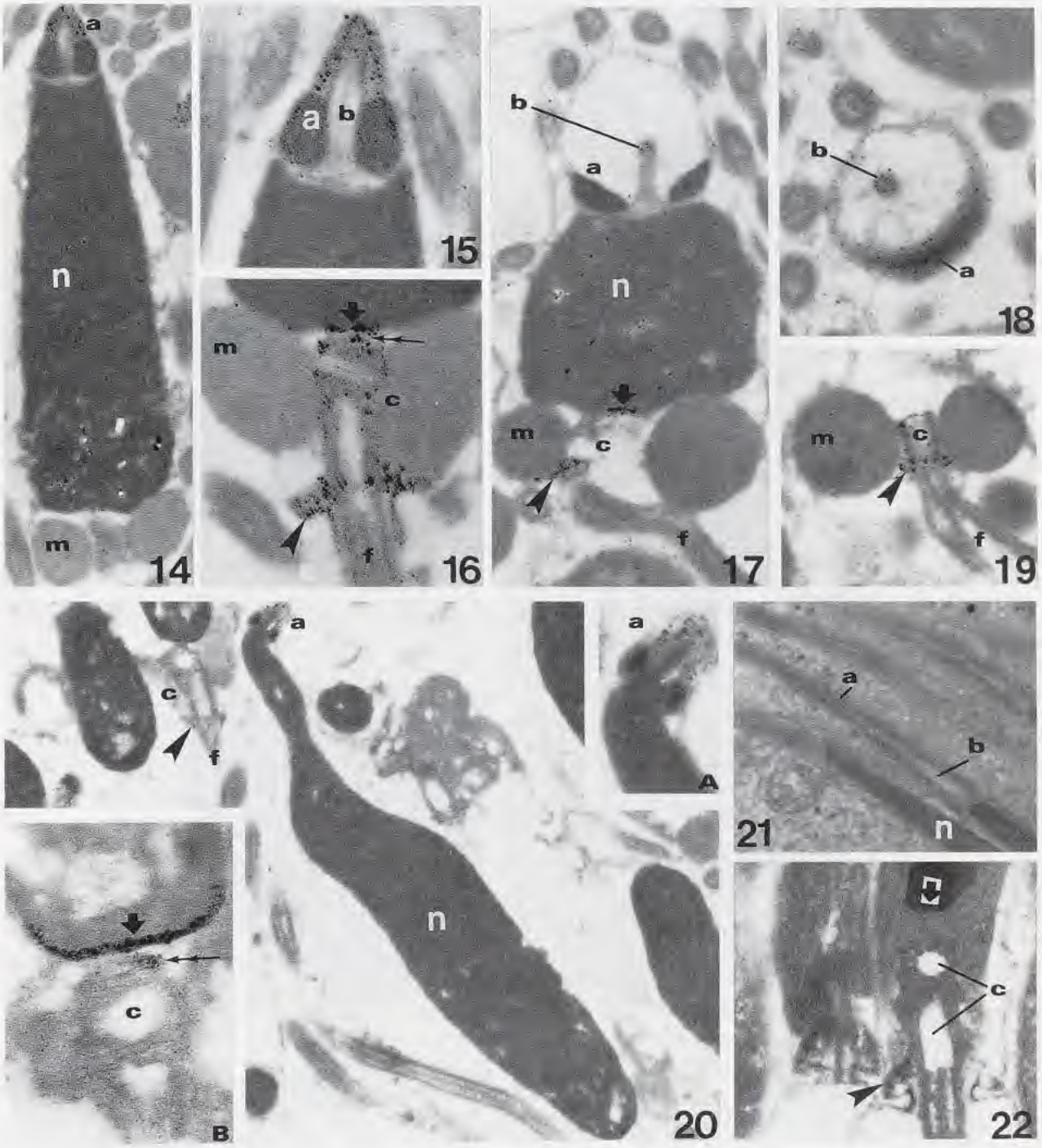
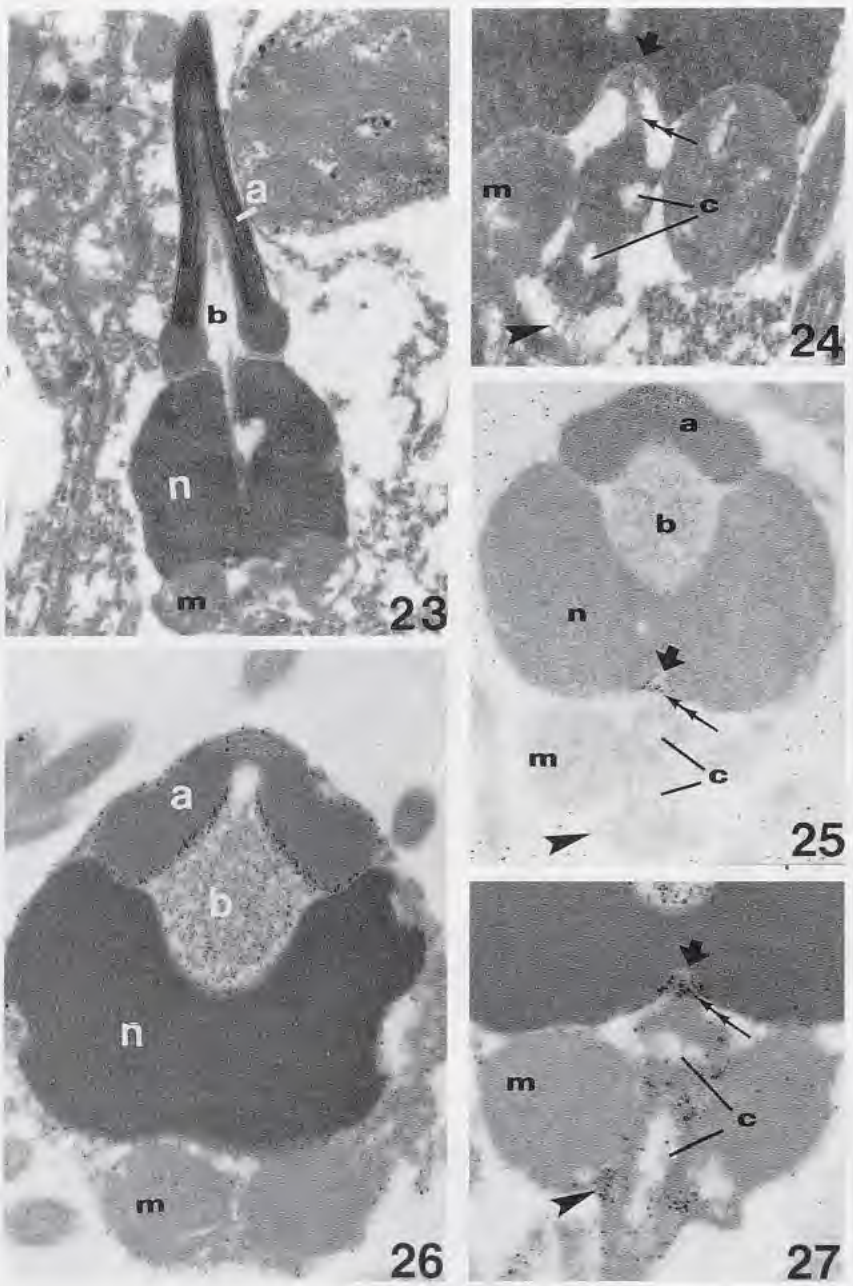
FIGS 14-22. — Veneroidea. a, acrosomal vesicle; b, subacrosomal material; n, nucleus; m, mitochondria; c, centrioles; f, flagellum. **14-16:** *Donax trunculus*. Silver stains the acrosomal vesicle (a), the centriolar fossa (arrow), the material that links the proximal centriole to the centriolar fossa (double arrow), the centrioles (c), and the pericentriolar complex (arrowhead). x 22 500; x 40 000; x 40 000. **17-19:** *Spisula solidissima*. Silver stains the dense component of the acrosomal vesicle (a), the insertion of the tip of the perforatorium into the acrosomal vesicle (b), the centriolar fossa (arrow) and the pericentriolar complex (arrowhead), and lightly stains the centrioles (c). x 25 700; x 36 200; x 27 600. **20:** *Cerastoderma edule*. Silver stains the acrosomal vesicle (a), the nuclear base (arrow), and the material that links the proximal centriole to the nuclear base (double arrows). Arrowhead, pericentriolar complex. x 20 000; inset A, x 46 000; inset B, x 80 000. **21, 22:** *Scrobicularia plana*. Silver stains the nuclear base (arrow) and the pericentriolar complex (arrowhead). x 16 900; x 47 400.

Source : MNHN, Paris



Source : MNHN, Paris



FIGS 23-27. — Pteriomorphia. a, acrosomal vesicle; b, subacrosomal material; n, nucleus; m, mitochondria; c, centrioles; large arrow, centriolar fossa; double arrows, material that links the proximal centriole to the centriolar fossa; arrowhead, pericentriolar complex. **23, 24:** *Mytilus edulis*. Silver stains the acrosomal vesicle. x 17 600; x 40 700. **25:** *Ostrea edulis*. Silver stains the tip of the acrosomal vesicle (a), the centriolar fossa (large arrow), and the material that links the proximal centriole to the centriolar fossa (double arrows). x 28 900. **26, 27:** *Crassostrea angulata*. Silver stains the tip of the acrosomal vesicle (a), the subacrosomal material (b), the centriolar fossa (large arrow), the material that links the proximal centriole to the centriolar fossa (double arrows), the centrioles (c), and the pericentriolar complex (arrowhead). x 30 900; x 39 900.

Source : MNHN, Paris

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The Use of Spermatozoal Ultrastructure in Phylogenetic Studies of Tubificidae (Oligochaeta)

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ABSTRACT

The influence of patterns of spermatozoal ultrastructure on hypotheses of phylogenetic relationships within the Tubificidae is examined on the basis of knowledge for species representing 15 different genera. A parsimony analysis of a combination of spermatozoal and conventional morphological characters supports that the Phallodrilinae, Limnodriloidinae and Tubificinae are monophyletic taxa, and that the Rhyacodrilinae is a paraphyletic group as currently defined. The mature spermatozoa of *Heterodrilus* spp., *Pectinodrilus molestus*, *Coralliodrilus rugosus*, *Smithsonidrilus hummelincki* and *Tubificoides amplivasatus* are described for the first time.

RÉSUMÉ

L'utilisation de l'ultrastructure des spermatozoïdes pour les études phylogéniques sur les Tubificidae (Oligochaeta)

L'influence des différentes organisations ultrastructurales des spermatozoïdes sur les hypothèses concernant les relations phylétiques à l'intérieur des Tubificidae est examinée, à partir de nos connaissances sur des espèces représentant quinze genres différents. Une analyse de parcimonie portant sur une combinaison de caractères des spermatozoïdes et de la morphologie conventionnelle indique que les Phallodrilinae, Limnodriloidinae et Tubificinae sont des taxons monophylétiques, et que les Rhyacodrilinae dans leur définition actuelle sont un groupe paraphylétique. Les spermatozoïdes mûrs de *Heterodrilus* spp., *Pectinodrilus molestus*, *Coralliodrilus rugosus*, *Smithsonidrilus hummelincki* and *Tubificoides amplivasatus* sont décrits pour la première fois.

The ultrastructure of spermatozoa has proved useful for phylogenetic assessment of higher taxa within the Clitellata (=Euclitellata *sensu* Jamieson [33]), particularly with regard to family level relationships in oligochaetes [34-35, 37, 40]. Spermatozoa are fairly uniform and distinctive within several clitellate groups [41], and their more general appearance supports a close relationship between clitellates and onychophorans [36, 37]. On the other hand, their ultrastructure may sometimes be used to distinguish species within the same genus [27, 44]. Evidence for great variation in the sperm ultrastructure of Tubificidae, a speciose group of aquatic oligochaetes, has been accumulated in recent years [24].

ERSÉUS, C., & FERRAGUTI, M., 1995. — The use of spermatozoal ultrastructure in phylogenetic studies of Tubificidae (Oligochaeta). *In*: JAMIESON, B. G. M., AUSIO, J., & JUSTINE, J.-L. (eds), Advances in Spermatozoal Phylogeny and Taxonomy. *Mém. Mus. natn. Hist. nat.*, **166** : 189-201. Paris ISBN : 2-85653-225-X.

Source : MNHN, Paris

The spermatozoa of tubificids, as well as those of all other clitellates, are characterized by a sequence of acrosome, nucleus, middle piece and tail (Fig. 2e). The acrosome contains an acrosome tube involving the other acrosome structures. The middle piece contains only the mitochondria. In the basal body region of the tail there is a prominent basal cylinder from which the two central tubules of the axoneme start. The axoneme shows a 9+2 arrangement and is characterized by the presence of some sort of accessory structure of the central apparatus [21], and a peripheral ring of glycogen granules. In members of the subfamily Tubificinae a double sperm line produces euspermatozoa and paraspermatozoa. (These different sperm categories have been called "typical" and "atypical", respectively, by one of us [2, 23], but here we adopt the terminology of HEALY & JAMIESON [30].)

Conventional morphological characters of tubificids, as well as of other aquatic oligochaetes, are few and many similarities are due to homoplasy, i.e., convergence or reversal [3, 13, 15]. This means that the support for monophyly of some groups, e.g. some tubificid subfamilies, is weak. Additional data, structural as well as molecular, are needed for a better understanding of the phylogenetic relationships within the Tubificidae.

In the present paper, parsimony analyses of both spermatozoal and conventional characters of 15 tubificid genera (Table 1), representing four subfamilies, are presented. The two sets of characters were run separately and in combination, to examine different impacts on the phylogenetic hypotheses. The majority of the spermatozoal data are from the literature, but for six marine species (representing the genera *Heterodrilus*, *Pectinodrilus*, *Coralliodrilus*, *Smithsonidrilus*, *Tubificoides*) the spermatozoa are described for the first time. When euspermatozoa as well as paraspermatozoa are present, both types are described, but in the parsimony analyses only euspermatozoa are considered.

MATERIAL AND METHODS

The material of *Tubificoides amplivasatus* was collected in muddy sediments in the Oresund, Denmark, in the summer of 1982. Specimens of *Heterodrilus pentcheffi* and *H. minisetosus*, and a single individual of *Pectinodrilus molestus* were found in subtidal sand at Long Key, Florida Keys, Florida, in October 1992. Additional material of *P. molestus*, and specimens of *Coralliodrilus rugosus* and *Smithsonidrilus hummelincki* were collected at subtidal and intertidal sites near Carrie Bow Cay, on the barrier reef off Belize in Central America, in March 1993. The material of *T. amplivasatus* was fixed in 3 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). The other material was fixed in a picric acid-glutaraldehyde-paraformaldehyde mixture following ERMAK & EAKIN [5]. After washing in the buffer and postfixation in similarly buffered 1% osmium tetroxide, the worms were dehydrated in a graded ethanol series and embedded in SPURR's resin. Sections were cut with LKB III and V, and observed under a JEOL 100XS electron microscope.

FITCH parsimony [25] analyses (i.e. multistate characters unordered) of tubificid taxa were performed using PAUP for Macintosh [43]. The branch-and-bound option (addition sequence: furthest) was selected. For analyses resulting in more than one equally parsimonious tree, strict consensus trees were calculated.

RESULTS

Description of new sperm types

Spermatozoa of Heterodrilus minisetosus *and* H. pentcheffi *(Rhyacodrilinae) (Figs 1b, g, l, 2c).* Spermatozoa were examined in the spermathecae. The two species have similar spermatozoa and will thus be described together. Only euspermatozoa are present, and spermatozeugmata are not formed. In both species, the acrosome is straight, about 0.6 µm long, 0.1 µm wide, with the vesicle, acrosome rod and secondary tube all withdrawn. The nucleus is apically straight, basally loosely twisted, and is followed by five twisted mitochondria, 1.4 µm long, 0.4 µm wide. The tail has two tetragon fibres, one much longer than the other.

Spermatozoa of Pectinodrilus molestus *(Phallodrilinae) (Figs 1c, f, 2e).* Spermatozoa were examined in the spermathecae. Only euspermatozoa are present, and spermatozeugmata are not formed. The acrosome is straight, 0.5 µm long, 0.1 µm wide. A secondary tube (if present at all)

Source : MNHN, Paris

is not visible. The nucleus is apically corkscrew-shaped, then gradually becomes twisted and is basally almost straight. Five twisted mitochondria follow, 2.5 µm long, 0.36 µm wide. The tail has tetragon fibres.

Spermatozoa of Coralliodrilus rugosus *(Phallodrilinae) (Figs 1a, k, o, 2d).* Spermatozoa were examined in the spermathecae. Only euspermatozoa are present and spermatozeugmata are not formed. The acrosome tube is corkscrew-shaped with an helical ridge making 1.5 gyres. The acrosome rod is indistinct. A secondary tube is not present. The nucleus is apically corkscrew-shaped and follows the pitch of the acrosome tube, then becomes twisted with a pitch increasing towards the base. The five mitochondria (1.2 µm long, 0.3 µm wide) are always surrounded by residual cytoplasm. The tail has tetragon fibres.

Spermatozoa of Smithsonidrilus hummelincki *(Limnodriloidinae) (Figs 1d-e, h, j, n, 2a-b).* Spermatozoa were examined at the ciliated male funnels as well as in the spermathecae. Euspermatozoa and paraspermatozoa are constantly present and easily distinguished. At the funnels the two types are randomly mixed, whereas in the spermathecae they are grouped in different spermatozeugmata, each containing only one sperm type. Euspermatozoa have an acrosome, 0.7 µm long, 0.15 µm wide, formed by a thin-walled, straight acrosome tube with a limen, a distinct secondary tube, a short rod and a partly withdrawn acrosome vesicle. The nucleus is apically twisted and basally straight. Four to five subspherical mitochondria form the middle piece. The tail shows a prominent central sheath. The paraspermatozoa have a shorter (0.36 µm) acrosome with the vesicle completely external to the tube, but with no other structure; a thin and irregularly outlined nucleus; two to four mitochondria characteristically swollen when the sperm are in the spermatheca, but not when at the funnels; a tail with a swollen plasma membrane when the sperm are at the funnels, but with apparently degenerating axonemes when in the spermatheca. The paraspermatozoa are fewer than the euspermatozoa.

Spermatozoa of Tubificoides amplivasatus *(Tubificinae) (Fig. 1i, m).* Spermatozoa at the funnels as well as in the spermathecae were examined. Both euspermatozoa and paraspermatozoa are constantly present. Spermatozeugmata are found in the spermathecae. They are composed of the two sperm types grouped together in the typical tubificine way [22]. We have no data on the acrosome of the euspermatozoa. The nucleus is apically twisted and basally straight. Three small ovoidal mitochondria separate the nucleus from the tail which has, at least in part, a prominent central sheath. The paraspermatozoa show a straight, empty acrosome tube, 0.5 µm long, 0.1 µm wide, with a small acrosome vesicle completely external to it. The nucleus is rectilinear, 2-3 µm long (which is shorter than that of the euspermatozoa). It has the shape of an elongated cone and is partly uncondensed. The two mitochondria are longer and larger than those of the euspermatozoa. The tail has a plasma membrane widely separated from the axoneme.

Parsimony Analyses

Taxa. Sperm ultrastructure has been reasonably well studied for 17 species of Tubificidae, representing 15 genera (Table 1). These 15 "genera", as characterized by their representatives studied, are regarded as the ingroup taxa. The outgroup is an hypothetical ancestor, the spermatozoal characters of which are in accordance with the plesiomorphic model suggested by JAMIESON *et al.* [40]. For the morphological characters, the ancestor is coded as a member the Phreodrilidae, a putative sister group of the Tubificidae [15].

Characters and character states. Two sets of characters are used, referred to as the "spermatozoal" and "morphological characters", respectively. All multistate characters are treated as unordered. The character states of all taxa are coded in Table 1. The character states are identical for *Heterodrilus pencheffi* and *H. minisetosus*, and for *Inanidrilus leukodermatus* and *I. bulbosus*.

Source : MNHN, Paris

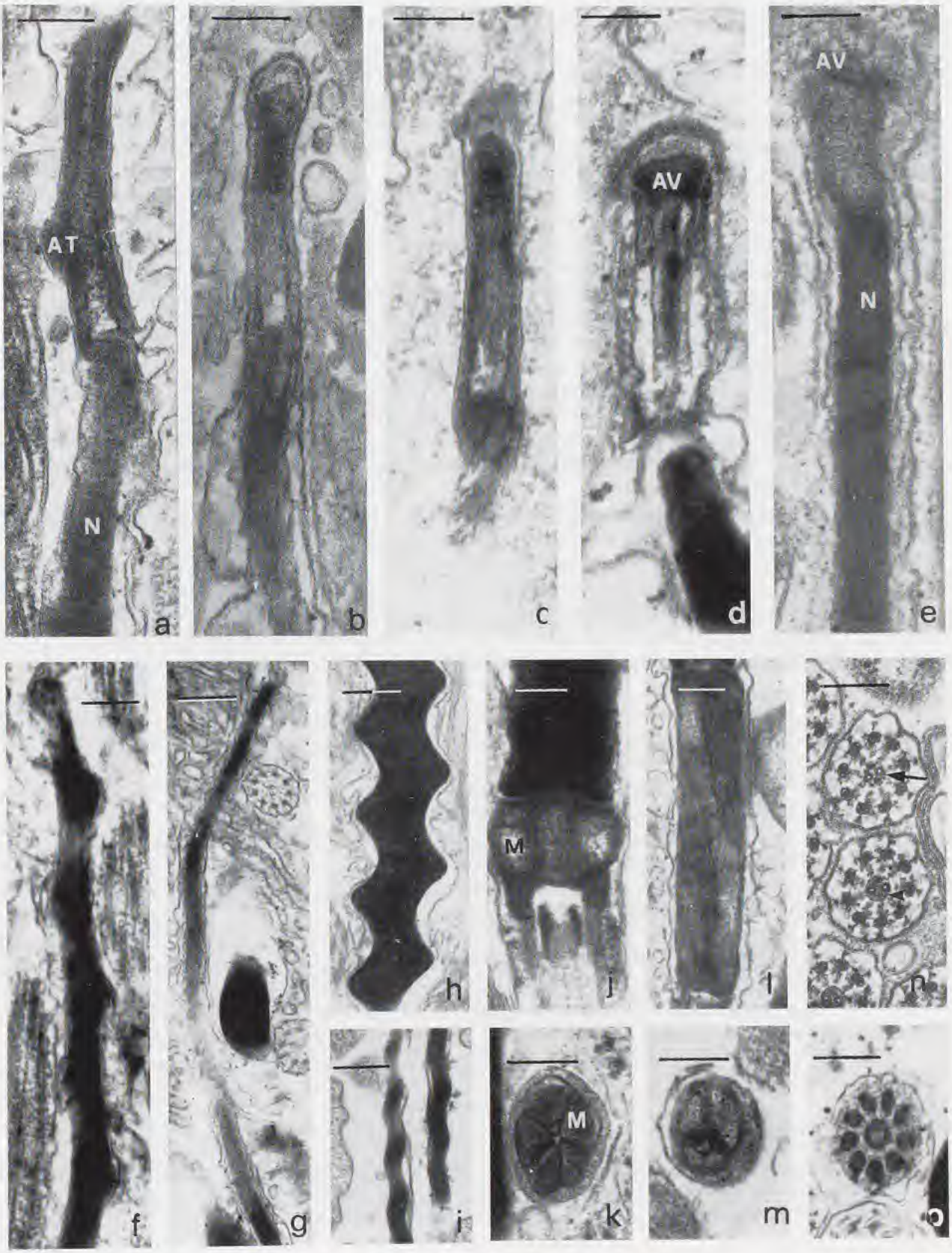
TABLE 1. — List of ingroup taxa included in the parsimony analyses. The genera are grouped according to their current subfamilial positions. [1] *Rhyacodrilus arthingtonae* is an enigmatic species, possibly not a natural member of *Rhyacodrilus*, but it does not belong to *Rhizodrilus* [19] as was suggested before [1, 24].

Taxon	Characters 1-20	Reference
Ancestor (hypothetical)	00000 00000 00000 00000	[15, 40]
Rhyacodrilinae		
Rhyacodrilus[1] *arthingtonae* Jamieson	00000 01000 ??111 00000	[32, 39]
Rhizodrilus russus Erséus	10110 11200 00111 00000	[16, 24]
Monopylephorus limosus (Hatai)	00010 11111 00101 00010	[20, 24]
Heterodrilus pentcheffi Erséus	00010 01010 11111 00010	[present study, 10]
Heterodrilus minisetosus Erséus		[present study, 10]
Phallodrilinae		
Bathydrilus formosus Erséus	10010 11210 01110 00310	[present study, 12]
Olavius planus (Erséus)	00010 11210 01110 10310	[8, 24]
Inanidrilus leukodermatus (Giere)	00010 11210 01110 10310	[24, 28]
Inanidrilus bulbosus Erséus		[9, 24]
Thalassodrilus prostatus (Knöllner)	01100 10110 12110 00310	[6, 24]
Pectinodrilus molestus (Erséus)	00010 11210 11110 00310	[present study, 14, 18]
Coralliodrilus rugosus Erséus	01010 11210 11110 00410	[present study, 17]
Limnodriloidinae		
Smithsonidrilus hummelincki (Righi & Kanner)	20011 01100 02100 01200	[11, 42]
Thalassodrilides ineri (Righi & Kanner)	10111 00010 01100 01200	[22, 42]
Tubificinae:		
Tubifex tubifex Müller	30010 01111 01000 00101	[2, 31]
Clitellio arenarius (Müller)	30010 01100 02100 00411	[24, 29]
Tubificoides amplivasatus (Erséus)	3???? ??101 02000 00101	[present study, 7, 24]

FIG. 1. — Character variation in tubificid spermatozoa (for interpretation of ultrastructural details, see Fig. 2). **a:** *Coralliodrilus rugosus*, longitudinal section of acrosome, bar 0.15 µm. **b:** *Heterodrilus minisetosus*, longitudinal section of acrosome, bar 0.15 µm. **c:** *Pectinodrilus molestus*, longitudinal section of acrosome, bar 0.15 µm. **d-e:** *Smithsonidrilus hummelincki*, longitudinal section of eusperm acrosome (d), and of nuclear tip and acrosome of paraspermatozoon (e), both bars 0.15 µm. **f:** *Pectinodrilus molestus*, corkscrew-shaped portion of nucleus, bar 0.2 µm. **g:** *Heterodrilus minisetosus*, apical, straight portion of nucleus, bar 0.2 µm. **h:** *Smithsonidrilus hummelincki*, twisted portion of eusperm nucleus, bar 0.3 µm. **i:** *Tubificoides amplivasatus*, twisted portion of two eusperm nuclei, bar 0.5 µm. **j:** *Smithsonidrilus hummelincki*, euspermatozoon, nuclear base (top), round mitochondria (centre), and basal body area with evident basal cylinder (bottom), bar 0.2 µm. **k:** *Coralliodrilus rugosus*, cross section of midpiece with five mitochondria (some residual cytoplasm present in the mature spermatozoon), bar 0.2 µm. **l:** *Heterodrilus minisetosus*, longitudinal section of midpiece, bar 0.2 µm. **m:** *Tubificoides amplivasatus*, cross section of midpiece with three mitochondria, bar 0.2 µm. **n:** *Smithsonidrilus hummelincki* euspermatozoon, cross section of tails at different levels, showing central apparatus with tetragon fibres (top), and prominent central sheath (bottom), bar 0.2 µm. **o:** *Coralliodrilus rugosus*, cross section of basal body area with basal cylinder, bar 0.2 µm.

Abbreviations in Figs 1 and 2: AR, acrosome rod, AT, acrosome tube; AV, acrosome vesicle; BC, basal cylinder; M, mitochondria in midpiece; N, nucleus; T, tail.

Source : MNHN, Paris



Source : MNHN, Paris

Spermatozoal characters

1. Spermatozeugmata: absent (0); present with only one type of spermatozoa (1); of two types present, one type formed by euspermatozoa, the other by paraspermatozoa (2); of one type formed by both euspermatozoa and paraspermatozoa (3).

2. Acrosome shape: straight (0); twisted or corkscrew-shaped (1).

3. Acrosome slenderness: length/width ratio less than 8 (0); greater than 8 (1). The slenderness and absolute length of the acrosome vary among species; by presenting the states as ratios the influence of absolute length is eliminated.

4. Acrosome vesicle withdrawal: ratio acrosome vesicle length/length of portion withdrawn into acrosome tube considerably greater than 2 (0); less than 2 (1). We accept the assumption [40] that an acrosome vesicle external to the acrosome tube is plesiomorphic. The ratio eliminates the influence of absolute length.

5. Acrosome tube: thick-walled throughout (0); at least in part thin-walled (1).

6. Secondary acrosome tube: present (0); much reduced or absent (1).

7. Acrosome rod (perforatorium): protuberant (anterior tip outside acrosome tube) (0); not protuberant (wholly located inside acrosome tube) (1).

8. Shape of nucleus, anterior portion: straight (0); twisted (1); corkscrew-shaped or flanged (2). Terminology according to FERRAGUTI *et al.* [24].

9. Shape of nucleus, posterior portion: straight (0); twisted (1). Nuclear shape is described as two characters, since different combinations of anterior and posterior shapes are present among tubificid spermatozoa.

10. Number of mitochondria: four or five (0); less than four (1). Four or five is a common number of mitochondria in "primitive spermatozoa" [26, 40].

11. Mitochondrial shape: straight and roundish-to-oval, length/width ratio not greater than about 1.5 (0); spiral and elongate, length/width ratio considerably greater than 1.5 (1).

12. Central axonemal apparatus: with prominent central sheath throughout flagellum (0); with tetragon fibres throughout flagellum (1); with prominent central sheath in anterior portion of flagellum, but tetragon fibres in posterior portion of flagellum (2). See [21].

Morphological characters.

13. Hair setae: present (0); absent (1). With reference to the condition in the outgroup Phreodrilidae, possession of hair setae has been interpreted as a plesiomorphic trait in the Tubificidae [15].

14. Penial setae: absent (0); present (1).

15. Coelomocytes: absent or few (0); numerous (1).

16. Alimentary system: present, body wall without symbiotic bacteria (0); absent, body wall with symbiotic bacteria (1).

17. Oesophagus (in segment IX): unmodified (0); modified, either bearing a pair of diverticula, or dilated with reticulate blood plexus (1).

18. Prostate glands: diffuse (0); solid, pedunculate, one per atrium (1); lobed, broadly attached, one per atrium (2); solid, generally pedunculate, two per atrium (3); absent (4).

19. Ciliation of atrial epithelium: weak (cilia restricted to particular, ciliated cells) or absent (0); dense (atria heavily ciliated throughout) (1). In *Smithsonidrilus*, the atrial ampullae are ciliated, whereas the atrial ducts lack cilia; thus this character is coded as (0) for *Smithsonidrilus*.

20. Penes: poorly developed or absent (0); well developed, pendent within deep penial sacs (1).

Results of parsimony analyses

Analysis using only morphological characters (Fig. 3a). This analysis resulted in a single, most parsimonious, tree with 14 steps and a consistency index (CI) of 0.786. It suggests that Rhyacodrilinae, Phallodrilinae and Limnodriloidinae are monophyletic taxa, but *Clitellio* appears

Source : MNHN, Paris

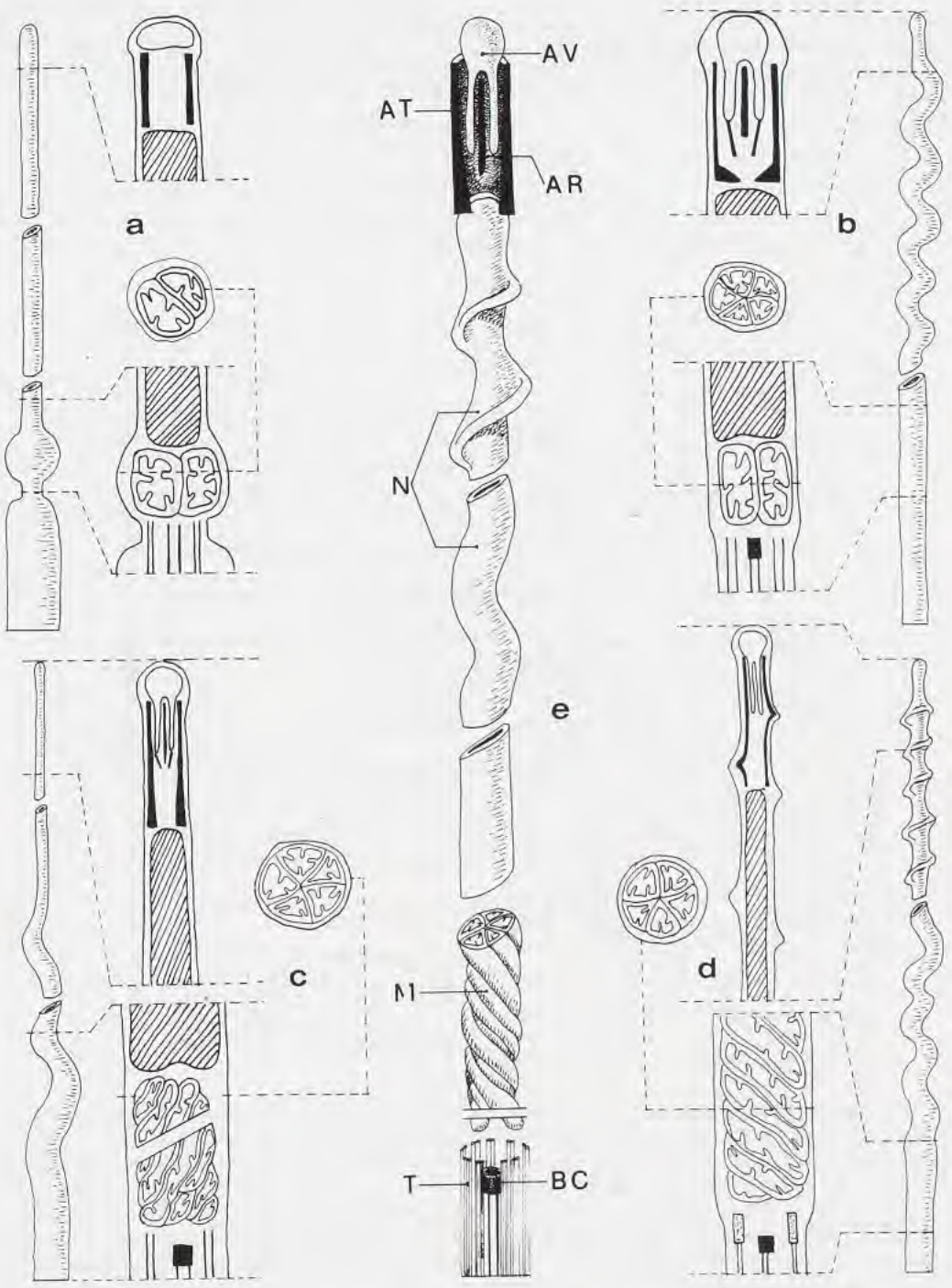


FIG. 2. — Schematic drawings of some tubificid sperm models. **a-b:** *Smithsonidrilus hummelincki*, paraspermatozoon (a) and euspermatozoon (b). **c:** *Heterodrilus pentcheffi*, euspermatozoon. **d:** *Coralliodrilus rugosus*, euspermatozoon. **e:** *Pectinodrilus molestus*, euspermatozoon. The plasma membrane has been omitted from the acrosome, mitochondria and tail of the *Pectinodrilus* spermatozoon (e), to show the internal structures. For abbreviations, see Fig. 1.

Source : MNHN, Paris

as the sister taxon of the Phallodrilinae rather than a member of the Tubificinae. The position of *Clitellio* is determined by its lack of prostate glands (state 4 of character 18), which is shared by *Coralliodrilus*, and the dense ciliation of its atria (character 19). For *Clitellio*, the coding of the latter character is based on a recent light microscopical study of *C. arenarius* [29]. Most other tubificines (including *Tubifex* and *Tubificoides*) are still assumed to have few cilia (if any at all) in their atria [15].

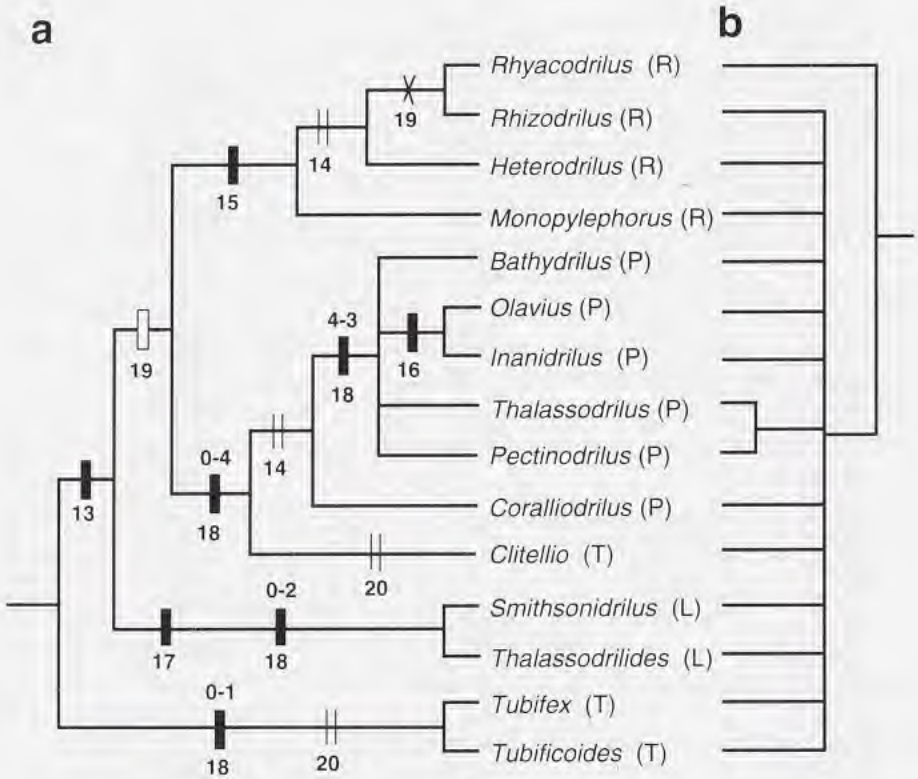


FIG. 3. — Results of parsimony analysis. **a:** The (one) most parsimonious tree for 15 taxa of Tubificidae, based on eight morphological characters (13-20). Length 14 steps; consistency index 0.786; retention index 0.893. Rooting at an hypothetical ancestor (see text). Subfamilial positions of taxa indicated in parentheses (*L*, Limnodriloidinae; *P*, Phallodrilinae; *R*, Rhyacodrilinae; *T*, Tubificinae). Numbers above symbols for multistate character refer to transformations of character states (e.g., *0-1* means "going from state 0 to state 1"). *Filled rectangle*, (unique) autapomorphy; *open rectangle*, autapomorphy that is followed by reversal further up the tree; *two parallel lines*, convergence; *cross*, reversal. **b:** Strict consensus tree of 45 equally parsimonious trees for 15 taxa of Tubificidae, based on spermatozoal characters (1-12).

Analysis using only spermatozoal characters (Fig. 3b). These characters yielded 45 equally parsimonious trees, all with 32 steps and a CI of 0.500. The congruence between these trees was very low, i.e, the spermatozoal characters proved more homoplasic than the morphological

Source : MNHN, Paris

characters. The only unequivocal suggestions from the sperm data are (1) that *Rhyacodrilus* has the most plesiomorphic spermatozoa known among the Tubificidae, and (2) that *Thalassodrilus* and *Coralliodrilus* are very closely related.

Analysis using both sets of characters (Fig. 4). When the spermatozoal and morphological characters were combined, the stability in tree topology increased considerably, although the CI (0.519) was barely greater than that of the trees based on only spermatozoal characters. The combined set gave three equally parsimonious trees, all with 52 steps, of which 36 emanate from the spermatozoal, 16 from the morphological characters. The trees differ only in the branching pattern of some phallodrilines, otherwise they all support (1) monophyly of each of Phallodrilinae, Limnodriloidinae and Tubificinae, (2) that the two latter are sister taxa, and (3) that the Phallodrilinae are cladistic members of the (otherwise paraphyletic) Rhyacodrilinae.

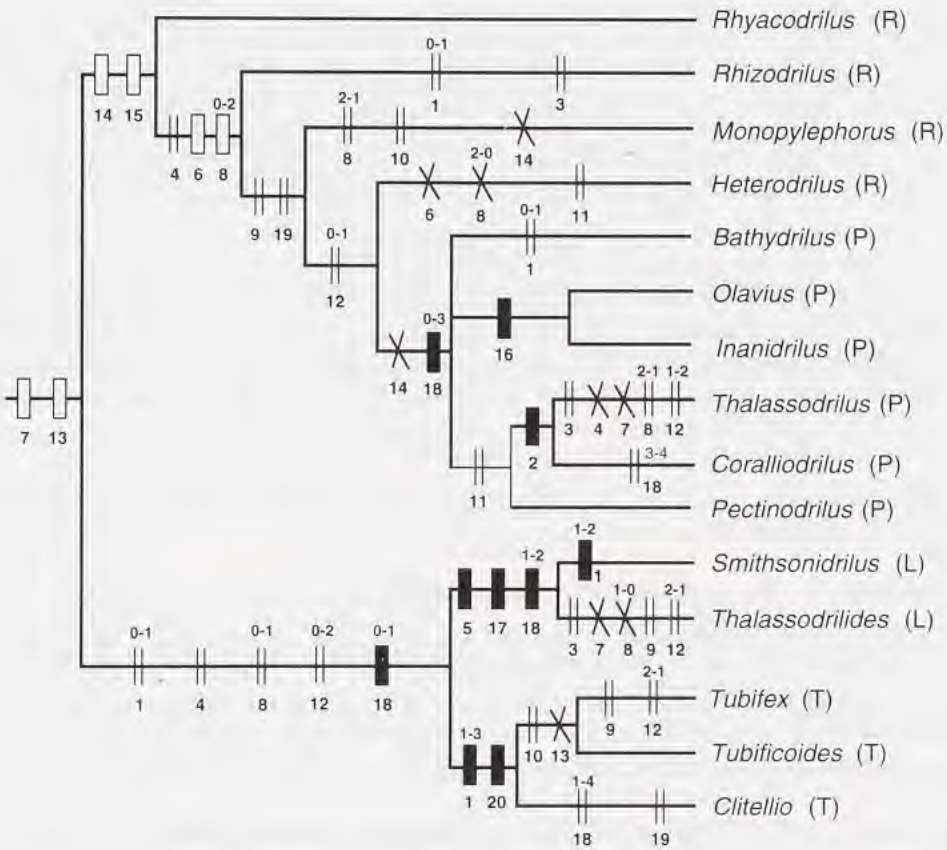


FIG. 4. — One of three equally parsimonious trees for 15 taxa of Tubificidae, based on twelve spermatozoal (1-12) and eight morphological characters (13-20). Length 52 steps; consistency index 0.519; retention index 0.638. Rooting at an hypothetical ancestor (see text). Branches shared by all three trees are indicated by bold lines; with another optimization of character 11, the group consisting of *Thalassodrilus* + *Coralliodrilus* and *Pectinodrilus* collapses. For other explanations, see Fig. 3.

Source : MNHN, Paris

DISCUSSION

The results of the parsimony analyses

The parsimony analyses revealed features of the character sets that were somewhat unexpected. The larger (spermatozoal) set could have been expected to yield the more stable trees, simply because on average there could be more character states to support each node in the trees. However, the poor congruence within the spermatozoal set made the morphological characters superior in terms of stabilizing tree topology.

With the two sets of characters combined, there is a trade-off between the congruence of the morphological data and the homoplasy of the spermatozoal data. Some traits of the tree based on morphology (Fig. 3a) are retained in the three trees based on all characters (Fig. 4), but there are also some changes. *Clitellio* now returns to the "tubificine" clade where it is normally classified, as the spermatozoal apomorphies (characters 1, 4, 8, 12) shared by *Clitellio* and *Tubifex/Tubificoides* (and partly by the limnodriloidines) outnumber the morphological ones shared by *Clitellio* and the phallodriline genus *Coralliodrilus* (characters 18, 19). The trees of the combined data set neither contradict the view that the Phallodrilinae, Limnodriloidinae and Tubificinae are monophyletic, nor that the Rhyacodrilinae are paraphyletic [15], although it should be noted that only a few selected genera of each subfamily are included in this analysis.

Two different hypotheses of subfamilial relationships within the Tubificidae, both based on conventional, morphological, characters, have recently been published. ERSÉUS [15] regarded the Phallodrilinae as an advanced subgroup within the "rhyacodriline" clade (a clade which also appears to contain the family Naididae, and possibly also the Opistocystidae), and this clade as a sister group of the rest of the family (Limnodriloidinae, Tubificinae and Telmatodrilinae). None of this is contradicted by the present study using the combined character sets; although the Telmatodrilinae are excluded from the present study. In the hypothesis presented by BRINKHURST [3], the Phallodrilinae is separated from the rhyacodriline clade and instead proposed to be the sister group of Limnodriloidinae-Tubificinae-Telmatodrilinae. This difference emanates from the fact that BRINKHURST's analysis included most aquatic oligochaete families in the ingroup, whereas the study by ERSÉUS focused on the Tubificidae and Naididae, using Phreodrilidae as the outgroup. BRINKHURST'S study implies that presence of hair setae is apomorphic when occurring in tubificid taxa, whereas this state turns out as a plesiomorphy in the present study as well as in that by ERSÉUS [15].

The relevance of sperm ultrastructure in studies of tubificid phylogeny

Both this and earlier studies [22, 24] show that tubificid sperm exhibit great variation in the ultrastructural details, sperm type differentiation, and formation of spermatozeugmata. Interestingly, the spermatozoa of species belonging to the same genus (although so far only investigated for *Inanidrilus* and *Heterodrilus*) are virtually identical, which indicates that spermatozoal characters are useful for the recognition of at least some genera within the Tubificidae.

Some spermatozoal features seem to be unique (aut)apomorphies of groups of genera (see Fig. 4): the twisted acrosome (character 2) of *Thalassodrilus* and *Coralliodrilus*, the partly thin-walled acrosome tube (character 5) of *Smithsonidrilus* and *Thalassodrilides*, and the double-line type of spermatozeugmata (character 1, state 3) of *Tubifex*, *Tubificoides* and *Clitellio*. However, a majority of sperm traits appear homoplasic. Much of the convergence is probably linked with the adaptive significance of some character states. For instance, very slender acrosomes (character 3) may have evolved at least three times in the Tubificidae; they are interpreted as independent transformations for *Rhizodrilus*, *Thalassodrilus* and *Thalassodrilides* (Fig. 4). The twisting of the nucleus (characters 8-9) seems to be convergent too, but here the most parsimonious hypotheses also imply reversal for these characters for some taxa (*Monopylephorus*, *Heterodrilus*,

Source : MNHN, Paris

Thalassodrilus and *Thalassodrilides*). The number of mitochondria has probably been reduced (character 10) at least twice, independently, for *Monopylephorus* and *Tubifex/Tubificoides*.

The withdrawal of the acrosome rod (character 7) is possibly an autapomorphy of the Tubificidae, but then this has become secondarily protuberant in *Thalassodrilus* and *Thalassodrilides*. Although the two latter genera clearly belong to different subfamilies, the spermatozoa of them exhibit several (apparently convergent) similarities: very slender acrosome, secondarily reduced (?) twisting of nucleus, and secondarily(?) protuberant acrosome rod. It is ironic that the type species of *Thalassodrilides* (*Limnodriloides gurwitschi* Hrabe) was once placed, for superficial morphological reasons, in *Thalassodrilus*; hence the name *Thalassodrilides* [see 4].

To conclude, tubificid spermatozoa provide a whole set of new ultrastructural characters, which are at least partly useful for phylogenetic assessments and, consequently, classification of taxa at different levels. However, the present study has shown that, in the Oligochaeta, the spermatozoal character patterns are complex and contain elements of convergence and probably also reversal, and therefore they should be used in tubificids only in combination with other information. Spermiocladistics, a term coined by JAMIESON [38], has proved useful for the reconstruction of phylogenies for many animal groups [37]. In many of the cases spermatozoal character patterns have been used to test, and indeed often to corroborate, hypotheses of phylogeny based on non-spermatozoal characters, or to propose new hypotheses of relationships. Thus, good congruence between spermatozoal and morphological data has been found at higher taxonomic levels in oligochaetes [34] and in the Clitellata, e.g. [21].

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Source : MNHN, Paris

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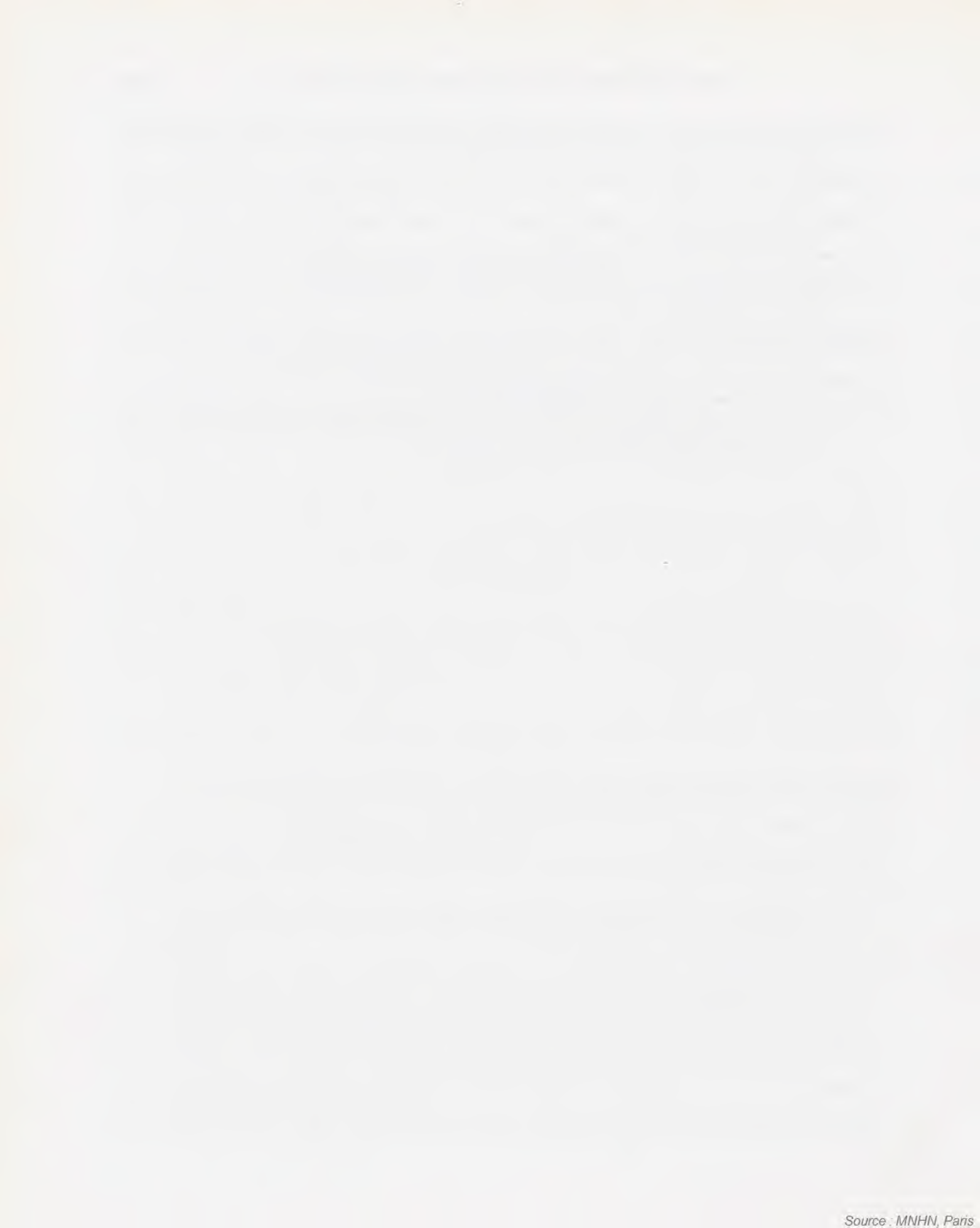

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Comparative Spermatology of Chelicerata: Review and Perspective

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ABSTRACT

Chelicerata have evolved separately from other arthropods since the Early Cambrian and thus provide many peculiarities aside of convergences realized within the framework of the arthropod concept. The present study describes the current knowledge on sperm cells of chelicerates. The most primitive types are found in the primarily marine taxa, in particular within the Xiphosura. Pantopoda have already quite derived sperm cells. In both groups, distinct character deviations are observable between spermatozoa of different taxa. These demonstrate the applicability of comparative spermatology for phylogenetic or systematic considerations. The most drastic modifications of the basic plan of spermatozoal structure are seen in the Arachnida. Representatives of all main taxa have now been studied, and their sperm cells are briefly discussed. In particular those of Araneae (spiders) and Acari (mites and ticks) have been extensively studied. The spider sperm cells are probably most interesting because of the various transport-forms such as encapsulated individual sperm cells (one cell per capsule: cleistospermia), capsules including several or numerous individual sperm cells (coenospermia and so called "spermatophores"), and, probably exceptional in the whole animal kingdom, capsules containing fused, syncytial sperm (synspermia). Acari all have aflagellate sperm of an extremely differing structure, which allowed the successful application of sperm ultrastructure for systematic purposes. In a subgroup of Acari it was possible to correlate alterations in the sperm structure with modifications in the genital systems and sexual behaviour. Thus, an evolutionary concept of sperm development in this taxon is suggested.

RÉSUMÉ

Spermatologie comparée des Chelicerata: synthèse et perspectives

Les Chelicerata ont évolué indépendamment des autres arthropodes depuis le début du Cambrien et donc montrent de nombreuses particularités ainsi que des convergences réalisées dans le cadre du concept des arthropodes. Ce travail décrit l'état actuel de nos connaissances sur les spermatozoïdes des Chélicérates. Les types les plus primitifs sont rencontrés dans les taxons marins de manière primitive, en particulier les Xiphosura. Les Pantopoda ont déjà des spermatozoïdes très dérivés. Dans les deux groupes, des déviations distinctes des caractères sont observées entre les spermatozoïdes de différents taxons. Ceci démontre que la spermatologie comparée peut être employée pour des considérations phylogénétiques ou systématiques. Les modifications les plus marquées du plan structural de base des spermatozoïdes sont rencontrées chez les Arachnida. Des représentants de tous les taxons principaux ont maintenant été étudiés, et leurs spermatozoïdes sont rapidement commentés. En particulier, les spermatozoïdes des Araneae (araignées) et des Acari (acariens, tiques) ont été étudiés de manière exhaustive. Les spermatozoïdes des araignées sont probablement les plus intéressants du fait des formes de transport variées telles que les spermatozoïdes encapsulés individuellement (une cellule par capsule: cléistospermie), des capsules contenant quelques ou de nombreux spermatozoïdes (coenospermie ou prétendus "spermatophores"), et, probablement exceptionnelles au sein du Règne Animal, des capsules contenant des spermatozoïdes fusionnés ou syncytiaux (synspermie). Les Acariens ont tous des spermatozoïdes aflagellés de structures

ALBERTI, G., 1995. — Comparative spermatology of Chelicerata: review and perspective. *In*: JAMIESON, B. G. M., AUSIO, J., & JUSTINE, J.-L. (eds), Advances in Spermatozoal Phylogeny and Taxonomy. *Mém. Mus. natn. Hist. nat.*, **166** : 203-230. Paris ISBN : 2-85653-225-X.

Source : MNHN, Paris

extrêmement différentes, ce qui permet d'utiliser avec succès l'ultrastructure des spermatozoïdes pour la systématique. Dans un sous-groupe des Acariens il a été possible de corréler les altérations de la structure des spermatozoïdes avec des modifications du système génital et du comportement sexuel. De ce fait, on suggère l'existence d'un concept évolutif du développement des spermatozoïdes dans ce taxon.

Spermatozoa of Chelicerata are now well known from the comparative point of view. Several authors have included spermatological characters in their considerations on phylogenetic systematics of Chelicerata [117, 136, 137], comparative spermatology in general [33], on comparative spermatology as a tool in phylogenetic systematics [138], on arthropod phylogeny [32] or on insect spermatology [75]. However, since not earlier than 1987 (Schizomida) [21] sperm cells of all main taxa had been described at least from one representative species, only an incomplete basis was available to these authors. Moreover, the many remarkable peculiarities shown by chelicerate spermatozoa require a separate study. This may stimulate further investigators to focus on spermatology of this interesting, diverse, and ancient group of arthropods, which has evolved separate from the other arthropods since the Early Cambrian [36, 120].

By far, the greatest structural diversity of spermatozoa is shown among the terrestrial and most species-rich Arachnida, which thus will dominate the following review.

OBSERVATIONS AND DISCUSSION

General aspects

Spermatozoa of Chelicerata represent the main categories of sperm structure distinguished today [32, 33, 35, 66, 74, 75].

The primitive sperm among chelicerates is only found in horse-shoe crabs (Xiphosura) [18, 29, 56, 124] and is characteristic of those taxa exhibiting aquatic fertilization (aquasperm) [sensu 74]. This type is classically characterized by a spherical head containing the acrosomal complex and nucleus with condensed chromatin, a middle piece containing a few relatively large mitochondria, and an elongate sperm tail representing the flagellum. The distal centriole is connected by an elaborate anchoring complex to the plasmalemma. It is already distinctly modified, however, in the Xiphosura [1, 18] (Fig. 1). The acrosomal complex is remarkable because of its very long acrosomal filament (perforatorium) which runs through the nucleus and is coiled around it. The chromatin is not as condensed as is usually the case in mature spermatozoa. The number of rather small mitochondria is quite high and a distinct middle piece is not always recognizable since the mitochondria may be scattered throughout the cytoplasm surrounding the nucleus in the spherical head region [18]. There is also no prominent anchoring complex [18, 56] and the axoneme of the flagellum may lack the central tubules (in the Indowest-Pacific species of *Tachypleus* and *Carcinoscorpius*) [18, 147].

The modified (derived) sperm (filiform-flagellate, biflagellate, or aflagellate to mention some obvious deviations from the primitive type) is characteristic of taxa exhibiting a modified fertilization. It is found thus in those aquatic (marine) taxa which have developed internal fertilization or are at least close to it and in terrestrial animals which depend on internal fertilization. Thus, in Chelicerata modified sperm cells are found in the marine Pantopoda and in Arachnida, which are primarily terrestrial animals.

In Pantopoda (Pycnogonida), the sperm of *Nymphon* species are still close to the primitive type (Fig. 1). However, an acrosomal complex is lacking and the nucleus is elongated. It is surrounded by longitudinally arranged microtubules which persist in the mature sperm cell. Similar to xiphosurans, chromatin condensation is only weak. The proximal centriole is reduced. There is a long flagellum in which considerable inter- and intraspecific variation in the axonemal pattern (9+0 to 18+0) occurs [48, 49, 52]. The sperm cells of *Nymphon* are motile. On the contrary, the elongate, immotile spermatozoa of *Pycnogonum littorale* are highly modified and

Source : MNHN, Paris

contain numerous longitudinally arranged microtubules (more than 1000) forming complex patterns. Aside of folded membranes (nuclear derivative?) found in the central part of the cell no further organelles were seen [49].

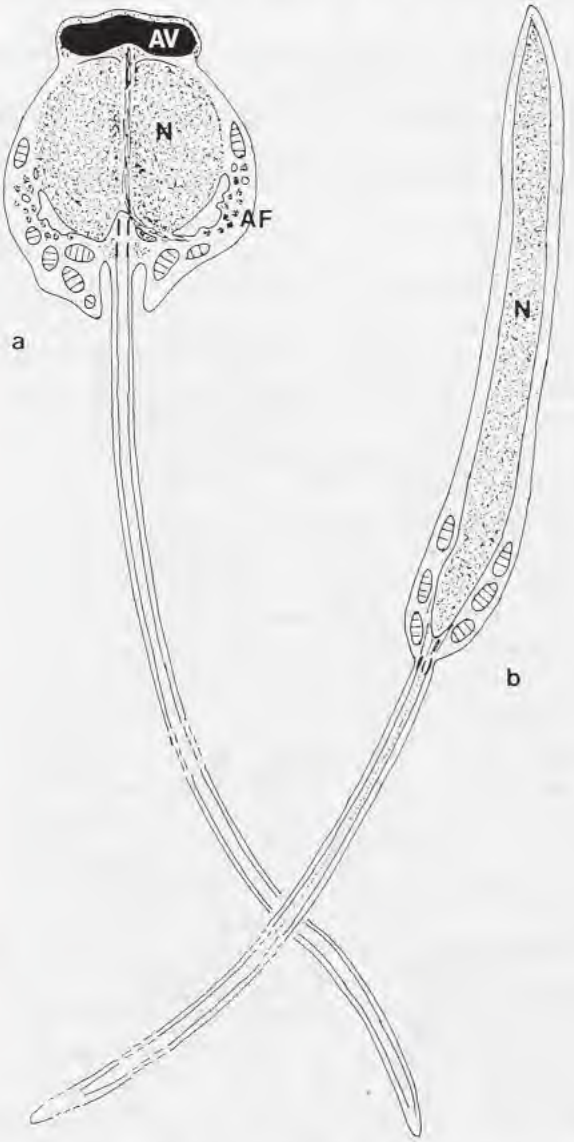

FIG. 1. — Diagrams of sperm of the primary marine chelicerates, Xiphosura and Pantopoda. **a:** *Tachypleus gigas* (Xiphosura) sperm are still close to the primitive type of sperm (aquasperm). The spermatozoon possesses a spherical head, a pronounced acrosomal complex, and a long flagellum. Note characters which deviate from the primitive sperm: very long acrosomal filament penetrating the nucleus and coiling around it, indistinct middle piece. In contrast to *Limulus polyphemus*, this species has a flagellum with 9x2+0 axoneme [18]. **b:** *Nymphon* sp. (Pantopoda). The sperm is further derived. It lacks an acrosomal complex, the head is elongated, the middle piece is indistinct, there is only one centriole [49, 52]. AF, acrosomal filament; AV, acrosomal vacuole; N, nucleus.

In Arachnida the main subtypes of modified sperm cells are distributed through the taxa as follows: filiform-flagellate (Scorpiones), coiled-flagellate (Pseudoscorpiones, Uropygi, Amblypygi, Araneae, Ricinulei), and aflagellate spermatozoa (Opiliones, Palpigradi, Solifugae, Acari) [4-8, 12, 13, 29, 21, 32, 33]. However, this classification gives only a rough impression of the diversity found within Arachnida: any conventional structure of sperm cells may be varied. Fig. 2 gives an impression of the various types of sperm depicting an example of each of the major taxa.

The extraordinary diversity may be first simply expressed by variations in magnitude. Sperm cells of Arachnida range between less than 2 µm (*Nicoletiella lutea*/Acari-Actinotrichida; *Siro duricorius*/Opiliones-Cyphophthalmi) [5, ALBERTI, unpublished] to 1000 µm in the argasid tick *Ornithodorus tholozani* (capacitated sperm cell; see below) [63].

Source : MNHN, Paris

The individual sperm cell may be of a very complex structure (e.g. Pseudoscorpiones; certain Acari-Anactinotrichida; Fig. 2) or may be rather simple (e.g. Solifugae; certain Acari-Actinotrichida) [5, 7] (Fig. 2). In the spider mite *Tetranychus urticae* (Acari-Actinotrichida), spermatozoa merely are comprised of a chromatin body, some cytoplasm, and a plasmalemma with tubular indentations [22].

However, primarily arachnid spermatozoa possess the same set of organelles which characterizes the ground plan of the metazoan sperm cell [33-35] (see above).

BACCETTI [32, 33] several times stressed the major evolutionary alterations in the arachnids: coiling of sperm cells and loss of flagellum.

In the following, modifications of conventional structures will be described at first, and secondly, new types of organization will be considered.

Acrosomal complex

The acrosomal complex typically is composed of an acrosomal vesicle (vacuole) and an acrosomal filament (perforatorium) (Fig. 3). The space between acrosomal vacuole and plasmalemma is occupied by a more or less distinct substance termed preacrosomal (also extraacrosomal or periacrosomal) material. The acrosomal filament is part of the subacrosomal material containing actin filaments which are highly ordered [124]. Another component appears as an amorphous substance and is termed an intermediate substance. This acrosomal complex is primarily present in all arachnid orders, probably with the only exception of Palpigradi, in which no acrosomal filament is found (however, only one species has been observed until now) [4]. Sometimes these conventional structures achieve a peculiar organization. In contrast to certain other scorpions in which the acrosomal complex is at the tip of the elongate nucleus, in the sperm of *Buthus occitanus* the very small acrosomal vacuole is located aside of the helical anterior part of the nucleus. The subacrosomal material (more strictly the intermediate substance) surrounds the whole tip of the nucleus [8], comparable to the subacrosomal cone found in certain Onychophora [74, 75]. A peculiar situation is observable in the opilionid *Nemastoma lugubre* [ALBERTI, unpublished] where subacrosomal material spreads between nuclear envelope and plasmalemma. Thus it apparently connects the latter to the nucleus and moulds the cell parallel to chromatin condensation and nuclear shaping [see also 81]. At the first glance, the situation found in certain Acari-Anactinotrichida (namely in the vacuolated type of sperm) [6, 9, 13] is quite similar as in the opilionid just mentioned. Here the acrosomal vacuole is developed into a flat cisterna growing from a central acrosomal plate [6, 41, 42]. This flat cisterna is connected to the plasmalemma by preacrosomal material and the shape of the cell obviously is influenced by this (Fig. 8e).

The preacrosomal material often contains filaments which are probably stabilizing the anterior region of the cell during the acrosomal reaction [124]. Such filaments were found in several Araneae [24, 26, 109] and in the micro whip scorpion *Schizomus palaciosi* [21] (Fig. 3b). In the latter an intricate paracrosomal lattice structure appears in the late stages of spermiogenesis as a transient structure (Figs 3d, 5). Regarding the various shapes of the acrosomal vacuole one extraordinary example was already demonstrated from the Acari-Anactinotrichida (Figs 2, 8a, c, e). Another well-known example is that of pseudoscorpions. In this group the acrosomal vacuole continues into a spiral band which surrounds the elongate smooth nucleus [40, 133] (Fig. 2). A peculiar sickle-shaped acrosomal vacuole was observed in Ricinulei [20] (Fig. 3c).

Finally it may be mentioned that certain taxa of Opiliones and Acari have lost the acrosomal filament (as *Prokoenenia wheeleri*, Palpigradi) [4] or are completely devoid of an acrosomal complex. Sometimes this occurs in the same genus: *Siro rubens* (Opiliones) with all acrosomal components) [80], *Siro duricorius* without acrosomal complex [ALBERTI, unpublished].

Source : MNHN, Paris

Nucleus

Whereas the corkscrew appearance of the nuclear region in Pseudoscorpiones is derived from the peculiar spiral band (acrosomal vacuole), the nuclei of certain scorpions and of Uropygi themselves exhibit a helical, in Amblypygi and basically also in Araneae a corkscrew shape (with sharp edges) [8, 24, 40, 76, 103, 107, 126, 133, 134] (Fig. 5). In the latter and also in the uropygid *Mastigoproctus giganteus*, the acrosomal filament spirals around the periphery of the nucleus enclosed in a nuclear canal [24, 103, 107] (Figs 3e, 4, 5). In Amblypygi and Araneae there is a tendency toward asymmetry of the nucleus which extends beyond the implantation fossa into a postcentriolar nuclear elongation [12, 26]. This asymmetry may be extremely developed in spiders of the genus *Tetragnatha* (Tetragnathidae) [12], in which the basis of the axoneme comes close to the acrosomal vacuole. On the other hand, the nucleus of *Pholcus phalangioides* (Pholcidae; Araneae) is nearly of radial symmetry, which is a derived characteristic as can be concluded by comparison with related taxa [24] (Fig. 5).

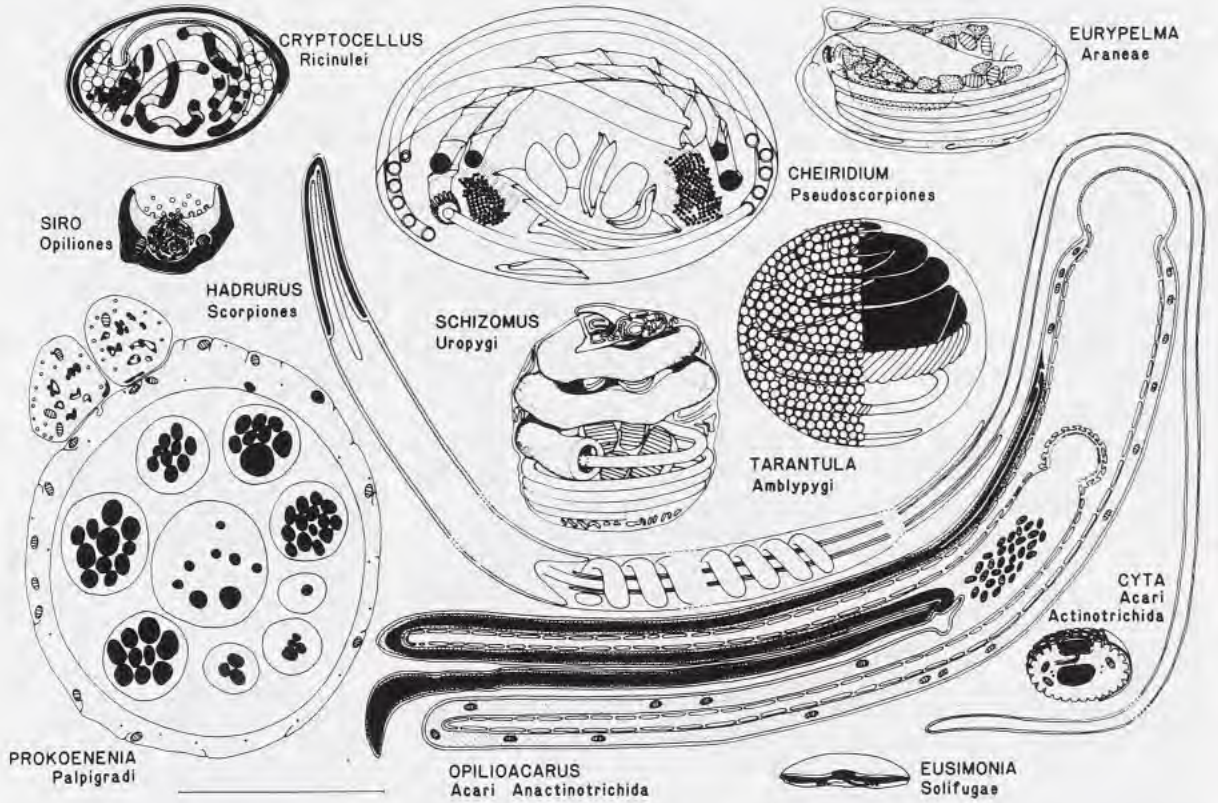

FIG. 2. — Synoptic view of spermatozoa of the terrestrial Arachnida (representatives of some taxa have secondarily invaded limnic and marine habitats). The spermatozoon of the scorpion is drawn in reduced length (full length: 275 µm). Spermatozoa of *Siro duricorius* and *Cheiridium museorum* [original], others [4-7, 9, 21, 26, 76, 77]. *Opilioacarus*: now *Neocarus*. Scale bar: 5 µm.

A comparable tendency as in *Tetragnatha*, probably even more peculiar, is observable in the ricinuleid *Cryptocellus boneti* [20]. In this species the axoneme is directly connected with the acrosomal vacuole, a situation - to the author's knowledge - not found anywhere else. However, the nucleus is not asymmetrical in contrast to *Tetragnatha*, though of extraordinary shape. The anterior part is a thin tube containing the acrosomal filament (Fig. 3c).

Source : MNHN, Paris

Even though these are only few examples, it is evident, that the nuclei may exhibit a considerable variety of shapes. In the flagellate spermatozoa, whether filiform or coiled, it is basically a large elongate organelle (Scorpiones, Pseudoscorpiones, Uropygi, Amblypygi, Araneae) [8, 20, 21, 76, 77, 103, 107, 126, 133, 134], which may even extend beyond the posterior end of the flagellum (*Cryptocellus boneti*) [20]. It is of interest, also from the viewpoint of functional spermatology, that a manchette of microtubules is observed only in some of these spermatozoa, namely in those of Uropygi, Amblypygi, Araneae and Ricinulei [20, 24, 26, 76, 126]. No manchette is found in Scorpiones and Pseudoscorpiones [40, 72, 77, 106, 133]. At this point it is not intended to continue the discussion on the possible function of a manchette of microtubules in nuclear shaping [see 21, 58, 127 for references]. It may only be mentioned that the microtubules exhibit alterations in their arrangement parallel to nuclear condensation in *Schizomus palaciosi* [21]. They follow exactly the sometimes aberrant configurations of the spermatid nuclei (e.g. in *Tetragnatha*) [12]. The manchette microtubules disappear at the end of spermiogenesis (see the pantopod *Nymphon*, however).

Another characteristic which largely varies within the Arachnida is the implantation fossa, a posterior region of the nucleus which usually contains the centrioles or their derivatives. The fossa may be a shallow, funnel-shaped indentation as in scorpions [8, 77], Uropygi/Thelyphonida [107], Amblypygi [76, 126] and Pseudoscorpiones [40, 134]. On the contrary, in Uropygi/Schizomida and many spiders the implantation fossa is deep, sometimes making the sperm almost a hollow tube (e.g. *Schizomus palaciosi*-Schizomida, *Pholcus phalangioides*-Araneae) [21, 24] (Figs 4, 5). Often dense material occupies the lumen of the fossa. This material may be homogeneous, granular (e.g. *Pholcus*) or may contain filaments or microtubules [92]. Within aflagellate spermatozoa an implantation fossa is observable only in opilionids. This is connected, in the genus *Siro*, with the transient appearance of a flagellum during spermiogenesis and is only a very shallow depression of the nuclear surface [80]. Nevertheless, in other opilionids a deep and broad invagination which contains the centrioles may occur [78, 81, 125].

In the aflagellate spermatozoa elongate, bulky, spherical, disc-, and bowl-shaped nuclei may be encountered (Fig. 2). It appears as if, after giving up the "basic plan" in early times, nearly any possibility of altering the shape of nucleus and cell has been realized. What is remarkable in particular with regard to the nucleus is the disappearance of the nuclear envelope in

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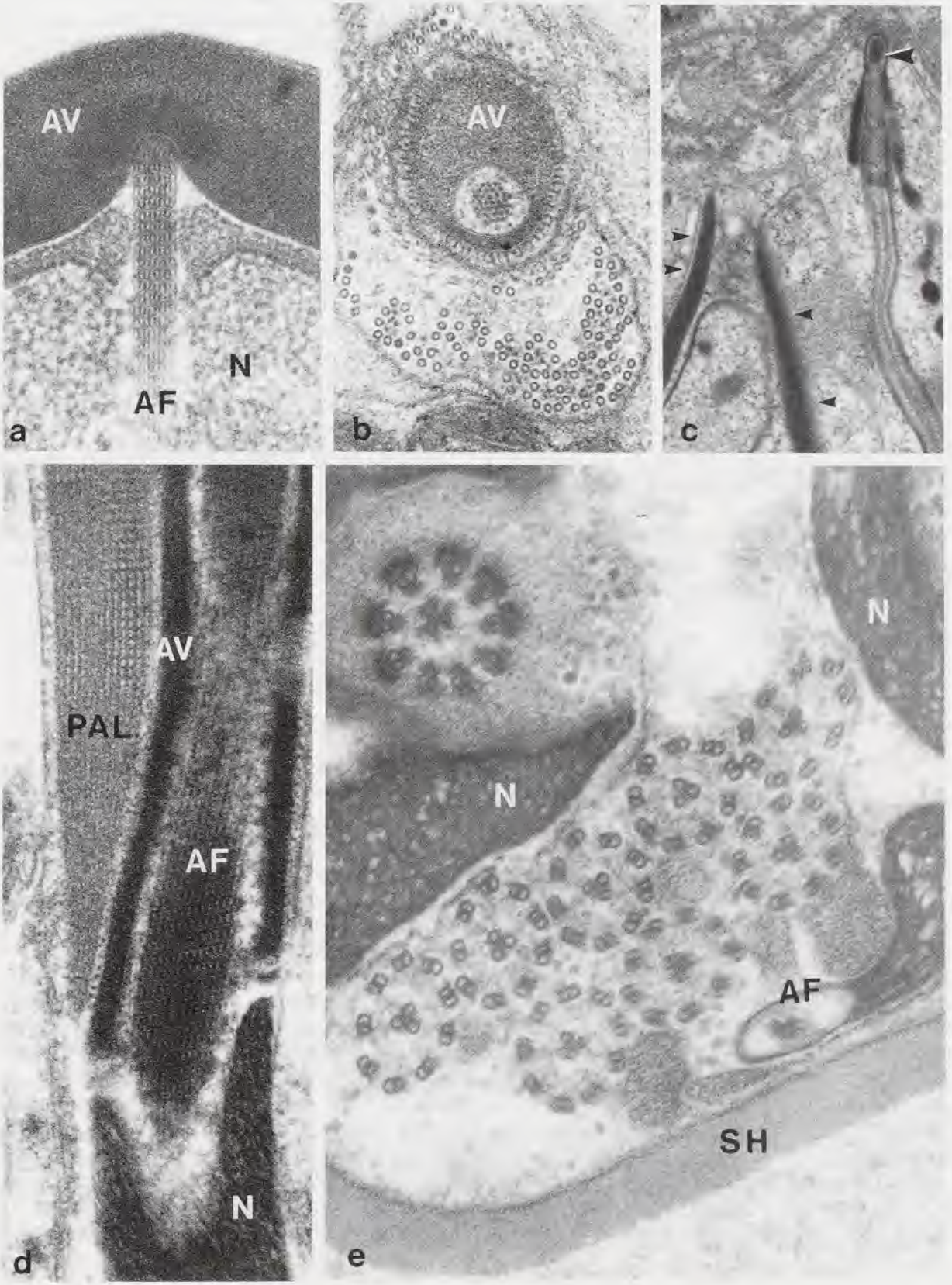
FIG. 3. — Details of spermatozoa of various Chelicerata. **a:** Acrosomal region of *Tachypleus gigas* (Xiphosura). Note acrosomal filament originating in the centre of a slight posterior indentation of the acrosomal vacuole and showing regularly arranged subfibres (actin) and nucleus with only loosely arranged chromatin [18]. x 53 000. **b:** Late spermatid of *Filistata insidiatrix* (Araneae). The cap-like acrosomal vacuole is sectioned transversely and the acrosomal filament composed of subfibres is shown. The acrosomal vacuole is surrounded by thin filaments. Note manchette microtubules [24]. x 49 500. **c:** Spermatid of *Cryptocellus boneti* (Ricinulei). The small, sickle shaped acrosomal vacuole is sectioned transversely (large arrowhead). It is surrounded by dense streaks. Note axoneme originating immediately behind the acrosomal vacuole (the nucleus lies parallel to the axoneme and is not shown in this figure). At left two of the dense plates which later ensheath the spermatozoon are seen (small arrowheads) (compare Figs. 2 and 5) [20]. x 28 500. **d:** Spermatid of *Schizomus palaciosi* (Uropygi-Schizomida). The basis of the long acrosomal vacuole, which is deeply indented from behind, rests on the nucleus. The acrosomal filament is rather thick and shows the same pattern of subfibres as in *Tachypleus gigas* (compare a). Note the paracrosomal lattice structure, which is only present during a short period in spermiogenesis [21]. x 63 000. **e:** Part of synspermium, i.e. the product of four fused spermatids, of *Segestria senoculata* (Araneae) showing basis of one axoneme and transverse sections through the coils of all four axonemes. Note 9x2 +3 axonemal pattern (synapomorphy of Megoperculata: Uropygi, Amblypygi and Araneae) and the absence of membranes separating the axonemes, what indicates the complete fusion between the four spermatids. A secretion sheath surrounds the synspermium [24]. AF, acrosomal filament; AV, acrosomal vacuole; N, nucleus; PAL, paracrosomal lattice structure; SH, sheath of secretion.

Source : MNHN, Paris



Source : MNHN, Paris

Solifugae and Acari-Actinotrichida at the end of spermiogenesis. In these taxa the acrosomal filament penetrates through the chromatin body and thus directly contacts the cytoplasmic matrix [6, 7] (Figs 6d, 8d). A similar situation is to the author's knowledge found only in Xiphosura [18, 56] (Fig. 1).

Midpiece

Since the work of ANDRÉ [28] the differentiation of the middle piece and chondriome in scorpions is known in detail. This is an example of extensive reorganization of mitochondria into elongate tubular structures (compare Fig. 2). No comparable elongation apparently occurs in Amblypygi and the early derived spider *Heptathela kimurai* (Mesothelae) [103] in which numerous mitochondria are arranged helically. Though quite different, in these three taxa a typical middle piece is established with mitochondria surrounding the flagellum or the axoneme respectively (Figs 4,5). In pseudoscorpions a different situation is found. The mitochondria are very elongate, thin tubular structures which are attached to the basis of the axoneme but they are otherwise situated in the cytoplasm of the coiled spermatozoon (see below) independently from the axoneme, where they establish a mitochondrial ring [40, 125] (Fig. 2). Contrary to scorpions, in pseudoscorpions the mitochondria are attached to the bases of the flagellum by an intricate structure (compare Fig. 2). In Uropygi no middle piece is encountered [21, 107, & PHILLIPS, 1986, pers. com.] (Fig. 5) as is the case in Ricinulei [20]. In Araneae, beside the primitive condition shown in *Heptathela*, mitochondria can be located without special arrangement (e.g. orthognath spiders, several labidognath spiders) [24, 26] or can be completely absent [93, 104] in the mature spermatozoa. Sometimes mitochondria pass through a "primitive" position in spermiogenesis being for some time located at the posterior end of the nucleus close to the basis of the flagellum (*Schizomus palaciosi*, several Araneae) [12, 21] (Fig. 5: *Schizomus*). In the aflagellate spermatozoa mitochondria are found in various positions sometimes "embedded" in the nucleus or chromatin body respectively (in the opilionid genus *Siro* and certain Acari-Actinotrichida) [6, 27, 64, 80, 142] (Fig. 7). Spermatozoa of some taxa may be devoid of mitochondria (e.g. *Tetranychus urticae*/Acari-Actinotrichida) [22].

Axoneme

The flagellum or axoneme respectively, if present, starts with two centrioles (Pantopoda are different in this respect; see above) arranged coaxially, an arrangement also found in the Xiphosura [18, 56] (Fig. 1). Thus this derived characteristic may be regarded as a synapomorphy of the chelicerates [136, 137]. Sometimes, however, the centrioles are arranged nearly orthogonally as in the mature spermatozoa of bird spiders [26]. Spermiogenesis reveals that this position is a derived arrangement in these spiders, probably caused by the coiling process, and follows a phase in which the centrioles are in tandem position. Even the original orthogonal arrangement is observable in the early stages of spermatogenesis [26].

One of the most interesting results from the viewpoint of comparative spermatology in Arachnology was the discovery of the 9x2 +3 axoneme in Araneae [103, 112], Uropygi [21, 107] and Amblypygi [76, 126] demonstrating the close relationship of these taxa with a spermatological criterion (Fig. 3e). However, even this apparently very stable characteristic (compared with the variability found e.g. in scorpions) [8, 72, 77] is altered in certain spiders (Linyphiidae) in which a 9x2 +0 axoneme occurs [12]. Thus this axoneme is by convergence similar to that of the xiphosuran genera *Tachypleus* and *Carcinoscorpius* [18, 147] and several scorpions [72].

Sometimes dense material surrounds the distal centriole thus probably establishing an anchoring complex (*Pholcus phalangioides*, *Dysdera* sp., *Segestria senoculata*; Araneae) [24] (Figs 3e, 6b). In spiders the number of tubules constituting the centrioles may be reduced and/or

Source : MNHN, Paris

dense fibres may be attached to the base of the axoneme. In *Cryptocellus boneti* (Ricinulei) the proximal centriole disappears during late spermiogenesis [20].

The only case where an aflagellate spermatozoon reveals relicts of an axoneme is represented by the cyphophthalmid *Siro* [80]. In these tiny opilionids a flagellate stage is observable during spermiogenesis.

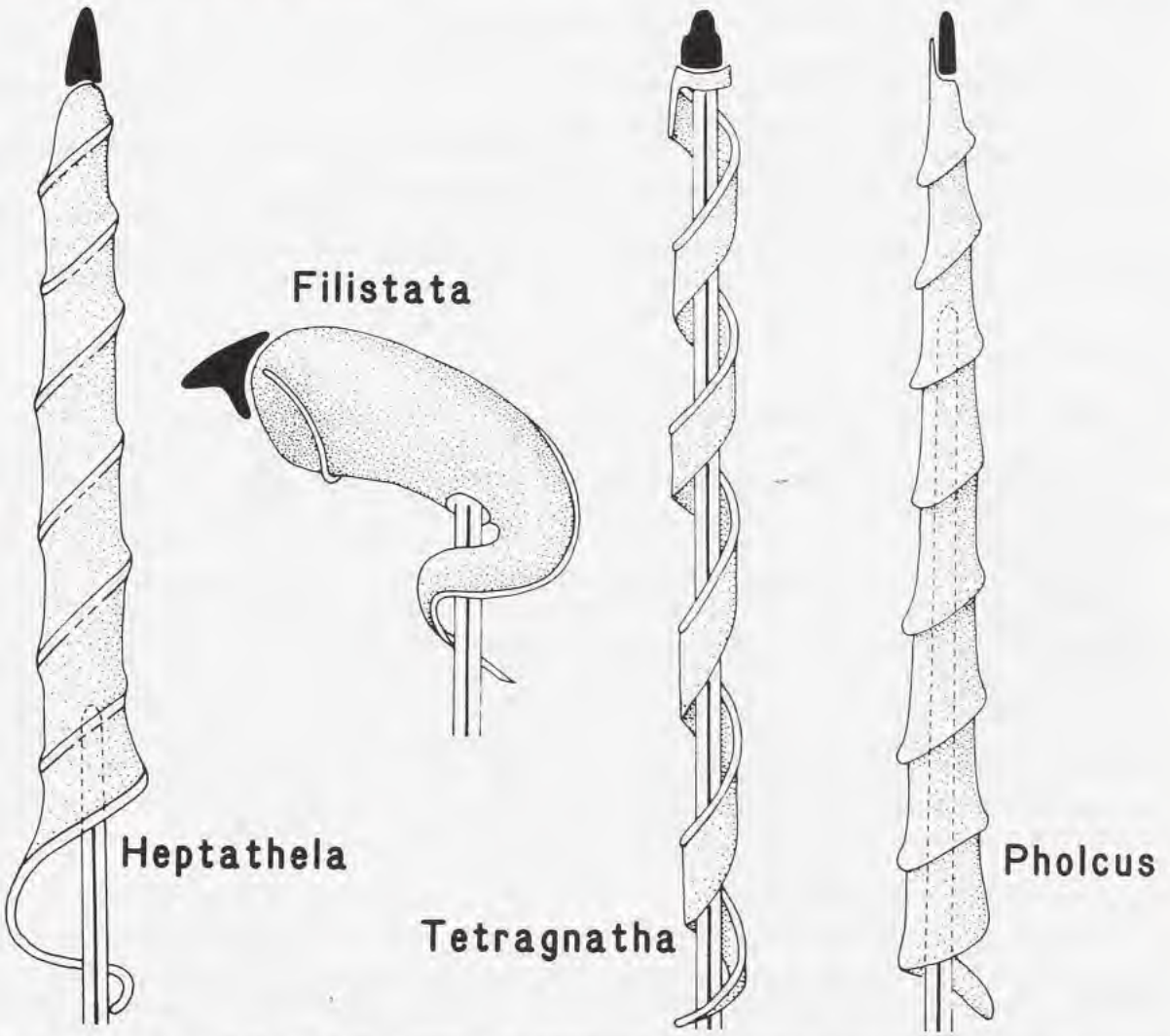


FIG. 4. — Different shapes of nuclei of spermatids of spiders and their relation to acrosomal complex and axoneme. **a:** *Heptathela kimurai* (Liphistiidae, Mesothelae). **b:** *Filistata insidiatrix* (Filistatidae, Opisthothelae). **c:** *Tetragnatha montana* (Tetragnathidae, Opisthothelae). **d:** *Pholcus phalangioides* (Pholcidae, Opisthothelae). The *Filistata*-type is the most common in spiders and may be close to the plesiomorphic type of Opisthothelae [12].

A peculiarity of scorpions and pseudoscorpions is the flagellar tunnel surrounding the axoneme and separating it from the middle piece mitochondria (in scorpions) and from the cytoplasmic matrix respectively (of the coiled cell in the pseudoscorpions) (Fig. 2). Whereas in the scorpions the flagellar tunnel comprises a distinct extracellular space, this is completely absent in pseudoscorpions owing to the close apposition of the membranes, which in early stages had delimited it. In scorpions and in some pseudoscorpions a dense cylinder surrounds the flagellar

Source : MNHN, Paris

tunnel. A flagellar tunnel is also observable during spermiogenesis of Uropygi, Amblypygi, Araneae and Ricinulei but disappears later [20, 21, 76, 107, 125]. Thus the axoneme is not surrounded by its genuine membrane in the mature spermatozoa of these taxa (Figs 3e, 6a, b).

It is important to note that the flagellar tunnel separates the flagellum from the mitochondrial sheath (in scorpions) or mitochondrial ring (in pseudoscorpions). In Amblypygi and the early derived spider *Heptathela*, in which a middle piece is still existent, the flagellar tunnel disappears before the mitochondria occupy their definite position (Fig. 5).

A flagellar tunnel appears also in connection with the transient flagellum of *Siro*. It is retained after the axonemal tubules have been withdrawn as a peculiar "crypt" containing numerous microvilli [80, ALBERTI, unpublished].

The flagellar tunnel may be derived from the cytoplasmic collar around the flagellar basis in xiphosuran sperm. No such structure occurs in Pantopoda (Fig. 1).

In addition to the diversity achieved in arachnids by variations of the basic sperm components "new" structures are introduced into this cell type resulting in new structural and functional possibilities and extraordinary peculiarities. These are dealt with in the following.

Vesiculation and vacuoles

Arachnid spermatozoa are very often rich in vesicles and cisternae, especially those belonging to the coiled-flagellate type. These vesicles - at least partly a result of the coiling process during which surface membranes are likely internalized - may be arranged very regularly under the plasmalemma (Uropygi/Thelyphonida, Amblypygi) [76, 107, 126] (Figs 2, 5). In Araneae and Ricinulei such vesicles and cisternae often contain dense material forming "dense streaks" (Fig. 3c). In certain Araneae vesicles fuse to large vacuoles [24]. In addition to these structures further vesicles are derived by an extended activity of the Golgi apparatus. In Ricinulei dark plates are formed early in spermatogenesis and contribute to an intracellular capsule enclosing the central components of the mature spermatozoon (Figs 2, 3c) [20]. In the palpigrade *Prokoenenia wheeleri* a huge vacuole dominates the large cell, which was previously thought to be a spermatophore (Fig. 2). The spermatozoa of certain Acari/Anactinotrichida develop during a very complex spermatogenesis a conspicuous vacuole bordered by a complex periphery bearing numerous "cellular processes" [5, 42, 63, 131] (Figs 2, 7, 8a,c). Perhaps the pouches of the ribbon sperm of related mites are derived from the vesicular precursors of the large vacuole characterizing the vacuolate type (Figs 8b) [5, 9, 13, 139-141].

Transport forms - sperm aggregates

Another aspect which was newly (or independently from non-arachnids) achieved during the evolution of spermatozoa of Arachnida is the establishment of secondary events. These transform elongate spermatozoa into the already mentioned coiled spermatozoa (Figs 2, 6a, b). In Uropygi and Amblypygi the coiling process starts from the posterior (flagellar) part of the cell resulting in a spherical mature spermatozoon. It is of interest to note that this coiling process is incomplete in the early derived spider *Heptathela kimurai* (Mesothelae) [89, 103]. Only the flagellum and posterior part of the nucleus are involved. In the bird spiders (orthognath spiders) the coiling is more complete, leaving only the acrosomal region projecting slightly from the otherwise more or less spherical cell [26]. Finally, in (most) labidognath spiders the whole cell including the acrosomal region is completely coiled or spherical again [12, 15, 24, 39, 92, 93, 104, 109]. If it is assumed that the spherical shape is the plesiomorphic state within the "Pedipalpi"-Araneae, the spiders demonstrate, probably, a nice example of a round about way in the evolution of their sperm cells. In Araneae the coiled cell is surrounded by an extracellular secretion sheath in different ways (Figs 3e, 6b and see below).

Source : MNHN, Paris

The coiling of the spermatid of Pseudoscorpions is a process which largely takes place in an extensive vacuole [134]. After completion of coiling the vacuole is transformed into smaller vesicles or cisternae (Fig. 2).

Extracellular material or secretions may loosely combine several spermatozoa to form aggregates (Scorpiones, Solifugae, Bdellidae/Acari/Actinotrichida) [7, 8, 10, 23] (Fig. 6c, d). In Opiliones/Cyphophthalmi the aggregates include dimorphic spermatozoa [80, ALBERTI, unpublished] (Fig. 6c). Such sperm aggregations in turn may be surrounded by a common sheath as in spiders of the families Theraphosidae and Filistatidae (coenospermia) [12, 24, 26, 37]. Rarely, such aggregates can deviate from the spherical shape. Thus, in Telemidae an elongate, rather complex tubular "spermatophore" is found [82]. A peculiarity observed to the author's knowledge only in a few Araneae (Dysderidae, Segestriidae, Scytodidae, Sicariidae) is occurence of syncytial spermatozoa (synspermia) [10, 12, 24] (Figs 3e, 6b). This fusion (or incomplete separation of spermatids) occurs already within the testis, whereas the coenospermia and spermatophores (Telemidae) are surrounded by a secretion sheath in the distal vas deferens as is the case in the majority of spiders which encase single sperm cells (cleistospermia) [12, 15, 24].

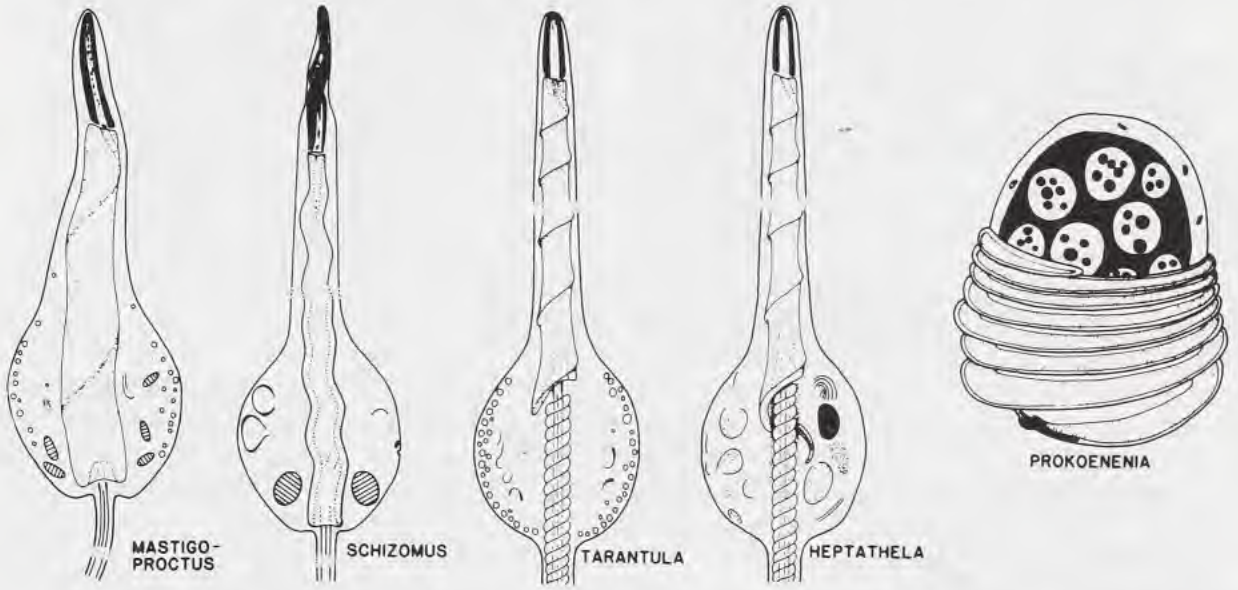


FIG. 5. — Late spermatids of: **a:** *Mastigoproctus giganteus* (Uropygi-Thelyphonida). **b:** *Schizomus palaciosi* (Uropygi-Schizomida). **c:** *Tarantula marginemaculata*. **d:** *Heptathela kimurai* (Araneae-Mesothelae). **e:** *Prokoenenia wheeleri* (Palpigradi). Note that the spermatid of the palpigradid deviates completely from those of the Megoperculata. A synapomorphy of Amblypygi and Araneae may be the postcentriolar elongation of the nucleus. The spermatids of *Schizomus*, *Tarantula* and *Heptathela* are drawn in reduced length [12].

Activation, maturation and capacitation

The spermatozoa of *Limulus polyphemus* remain immotile until they come into contact with a component which is released from the egg layer [44].

Spermatozoa of many taxa undergo certain modifications after leaving the male by which they achieve the capacity to fertilize the oocyte (capacitation) [35]. These transformations may be restricted to the molecular level as in mammals or may involve distinct morphological alterations [102, 130]. It is evident that these processes have to occur in a taxon-specific way since the sperm cells have to cope with a specific environment. In terrestrial animals this is usually the female genital tract. Exceptions are species with dermal insemination such as some Onychophora [94,

Source : MNHN, Paris

118, 119] in which the sperm cells migrate through the body cavity. In many arachnids the mentioned transformations occur in a rather drastic way. However, these events are poorly investigated. The transport form in which the sperm is transferred to the female is changed into the fertile, active sperm.

From Araneae and Pseudoscorpions it is known that spermatozoa uncoil in the female [37, 40] and only then are fertile [45]. Probably these events are facilitated by the mentioned vesicles and vacuoles which may be integrated into the plasmalemma when elongation of the cell occurs. This process is well known and very intricate in the vacuolated sperm cells of ticks and some mites (Acari/Anactinotrichida). The shape of the cell is completely changed by a turning-inside-out-process which brings the cellular processes to the surface of the cell [13, 41, 42, 63, 101, 113, 131]. After that, the sperm cells are capable of several kinds of motion [60, 101] (see Figs 2, 7, 8a, c for cells prior to capacitation).

In ticks, and probably also in anactinotrichid mites with vacuolate spermatozoa, a further transformation of the cell occurs prior to insemination. A posterior portion of the sperm cell which is invaginated into the cytoplasmic column (inner core), forming a so-called acrosomal canal in several species, is also evaginated and contains the nucleus [63, 113]. It has been suggested that only this nucleus-containing part of the capacitated spermatozoon enters the oocyte [101, 113].

In the ribbon spermatozoa of a related mite, *Varroa jacobsoni*, the spherical cell transforms into an elongate cell with completely new appearance and structures which are probably related to the achievement of motility [17].

In actinotrichid mites, e.g. spider mites (Tetranychidae), spherical spermatozoa become irregular and possess filaments in cell processes, probably enabling amoeboid movements [13, 16, 22, 98].

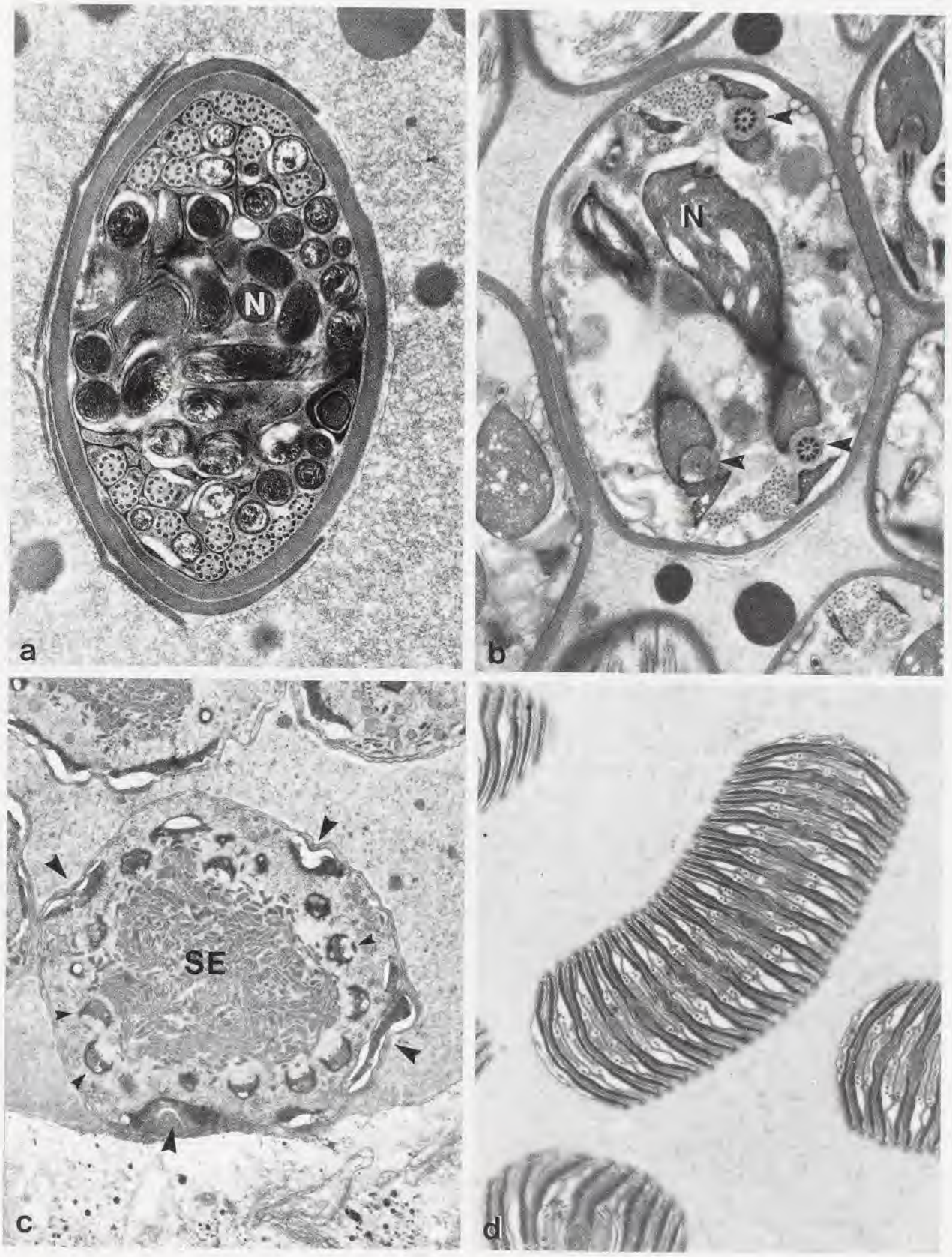
Acrosomal reaction

The acrosomal reaction of *Limulus polyphemus* is a classical subject of study in spermatology and has been described in detail [29, 47, 124]. Upon contact of the sperm cell with the egg, exocytosis of the acrosomal vacuole occurs. The contents of the vacuole ensures attachment to the egg. In about 4 seconds, a 60 µm long process grows out of the anterior end of the cell (so called true discharge). The process contains the acrosomal filament (perforatorium) which is uncoiled and remains covered by a membrane (the posterior membrane of the acrosomal vacuole to which material derived from the nuclear envelope is continuously added). The elongating process rotates, which helps the process to penetrate the extracellular layers of the egg. According to [47, 129] the driving force which creates the elongation of the process is the consequence of an alteration in the twist of the actin filaments within the acrosomal filament (compare Figs 1a, 3a).

No other chelicerate sperm cell has been investigated with regard to its acrosomal reaction. Because of the structural similarity of the acrosomal complexes of many arachnids it can be assumed that the main events are similar to those known from the xiphosuran (Fig. 3). However, the numerous peculiarities indicated above urgently need a detailed investigation.

FIG. 6. — Transport forms of spermatozoa in Arachnida. **a:** *Cryptocellus boneti* (Ricinulei). The coiled sperm cell is encysted. The cyst wall consists of two dense plates which are intracellular products (see Fig. 3c). Additional secretions are added in the vas deferens [20]. x 19 000. **b:** A synspermium of *Segestria senoculata* (Araneae). Arrowheads point to the bases of three (of four) axonemes [24]. x 13 250. **c:** A sperm ball of *Siro duricorius* (Opiliones-Cyphophthalmi) consists of peripherally arranged infertile sperm cells (large arrowheads) and smaller, cup-like fertile spermatozoa (small arrowheads). In the centre of the ball conspicuous secretions are seen (compare Fig. 2 and [80]). x 3 350. **d:** Pile of 32 spermatozoa arranged in 16 pairs in *Eusimonia mirabilis* (Solifugae) [7]. x 7 500. N, nucleus; SE, secretion.

Source : MNHN, Paris



Source : MNHN, Paris

Phylogenetic and systematic considerations

Sperm cells of Chelicerata are evidently very diverse and the taxon-specificity of these cells offers the possibility to use characteristics of these cells for taxonomic purposes [5, 6, 9, 12, 13, 32, 33, 35, 138].

Among the xiphosurans interesting features have been shown already when comparing the only Atlantic species (*Limulus polyphemus*) with the three Indowest-Pacific taxa (*Tachypleus gigas*, *T. tridentatus*, *Carcinoscorpius rotundicauda*). Apart from some deviations in the shape of the acrosomal vesicle, arrangement of mitochondria and acrosomal filament, the most obvious difference is the lack of the central microtubules in the axoneme of the Indowest-Pacific species. This is a synapomorphy of these taxa and demonstrates the result of a long independent evolution [18, 147].

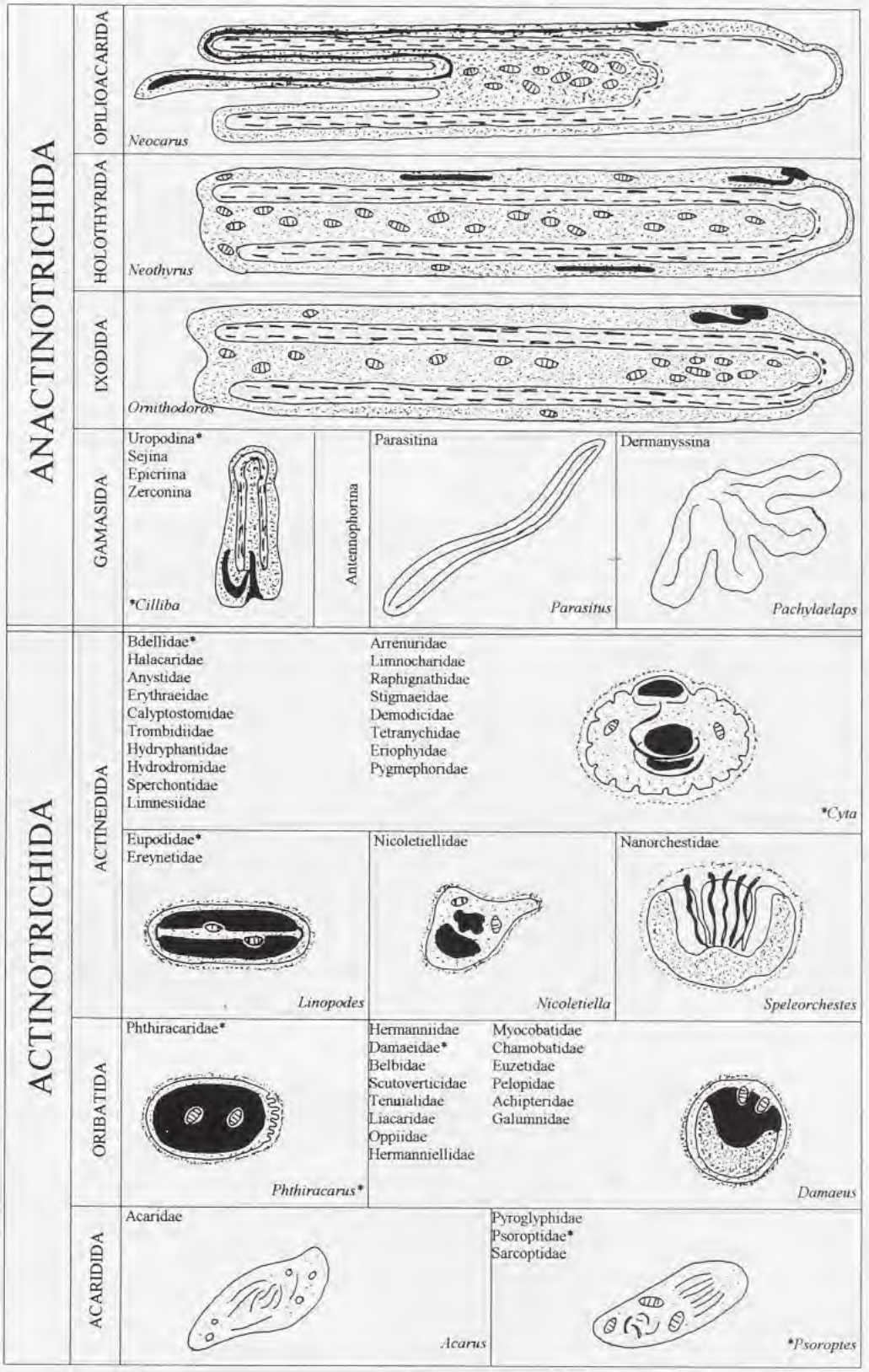
The few studies on Pantopoda have shown considerable differences between the two genera investigated (see above) which corroborates the view that Pycnogonidae is the more derived taxon compared with Nymphonidae [84, 97]. The axonemal pattern may be used to ascertain the validity of certain species (e.g. *Nymphon gracile* and *N. rubrum*) as separate taxa [52].

Within the Arachnida, only in the Opiliones [78, 81, 125] and Acari [5, 6, 9, 13] have systematic considerations been undertaken on the basis of a sufficiently wide range of taxa. Some encouraging aspects emerged also from the recent studies of "Pedipalpi" and Araneae [14, 21, 24, 26, 76, 82, 92, 126].

All of these sperm cells are, despite their wide range of complexity, capable of transferring the male's genetic information to the female germ cell. It is very challenging trying to trace the evolution of the sperm cell through the branching phylogenetic tree looking for a more comprehensive understanding of the alterations of this cell type. It is obvious that this is a long lasting project which leaves much work for the future. Arachnida represent a very old taxon, so there are not only the usual gaps in knowledge (which could be closed by future research) but also those caused by extinction of large taxa [36, 70, 86, 105, 136, 137]. Thus the extant major taxa of Arachnida are quite isolated from each other. Spermatology may provide a tool to bridge gaps in taxonomic understanding between these extant taxa. Furthermore it has to be kept in mind that convergences may also occur at the cellular level. Some examples were already mentioned: displacement of axonemal base to the anterior (*Cryptocellus*/Ricinulei, *Tetragnatha*/Araneae), 9x2 +0 axoneme (*Tachypleus*, *Carcinoscorpius*/Xiphosura, Scorpiones in part, Linyphiidae/Araneae), deep implantation fossa (*Schizomus*/ Uropygi, some Araneae such as *Pholcus*), helical or corkscrew appearance of sperm head (*Buthus*/Scorpiones, Pseudoscorpiones, "Pedipalpi"- Araneae). (In Araneae, this corkscrew appearance in *Pholcus phalangioides* is profoundly different from all other spiders and is thus probably an autapomorphic peculiarity of this taxon) [12, 24] (Fig. 4). Most likely another convergence is demonstrated by the shaping of the spermatid under the influence of "acrosomal" material (*Nemastoma*/Opiliones, vacuolate type of sperm/Acari-Anactinotrichida) (Fig. 8e). Different types of "envelopes" are found in the coiled sperm: extracellular ones (Pseudoscorpiones, "Pedipalpi", Araneae) and intracellular ones (*Cryptocellus*/Ricinulei) (Figs 3e, 6a,b). Some of these convergences are easily recognized by fine structural analysis or observation of spermiogenesis, others need comparison with related taxa on a wider scale.

FIG. 7. — Sperm types in Acari. Asterisks indicate genera and corresponding higher taxa [modified from 13]. Spermatozoa of Antennophorina are probably intermediate between vacuolated type and ribbon type [ALBERTI & BLASZAK unpublished]. Note that spermatozoa of Actinedida exhibit a diversity which can hardly be depicted here (compare [6, 13]). See also Fig. 9 with respect to Gamasida.

Source : MNHN, Paris



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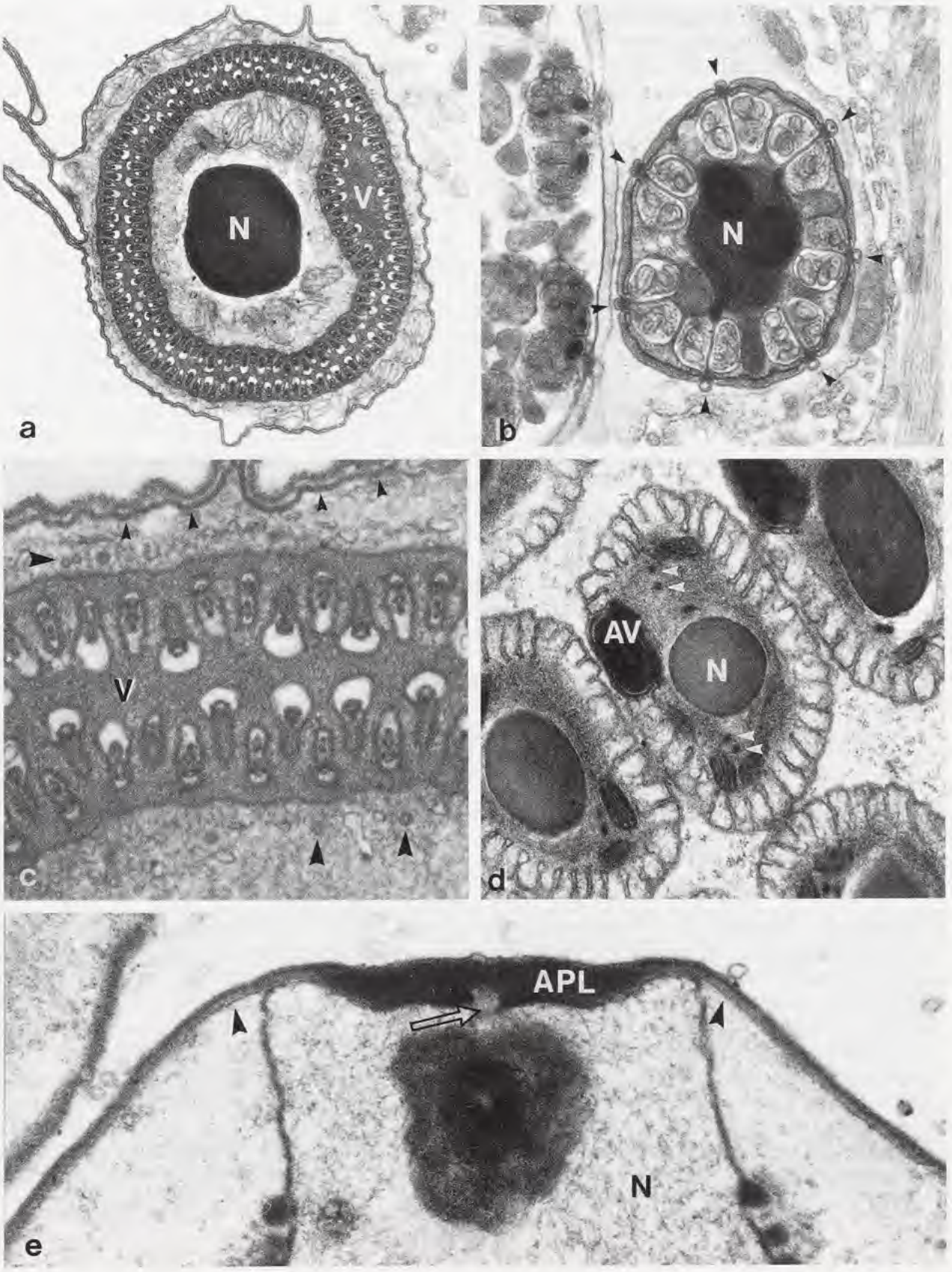
Thus spermatology may at least shed some new light on systematical interpretations and may induce a reconsideration of certain traditional ideas. Some examples of relationships which were suggested previously and which could be viewed at in the light of the new spermatological findings are: Pseudoscorpiones-Scorpiones [148]; Pseudoscorpiones-Solifugae [69, 114, 117, 136, 137]; Uropygi-Acari [111]; Solifugae-Acari/Actinotrichida [67, 149]; Acari/Anactinotrichida -Acari/Actinotrichida [83, 53, 91, 117, 136, 137]; Opiliones/ Ricinulei-Acari [30, 88]; Opiliones-Acari [83, 108, 114]; Ricinulei-Acari [53, 91, 117]; Ricinulei-Acari/Anactinotrichida [69]; Pedipalpi-Palpigradi [83, 110]; Schizomida-Palpigradi [38, 132]; Palpigradi-Acari/ Actinotrichida [69]. In some cases it was already possible to bridge some gaps or solve open questions.

The well known example of the 9x2+3 axoneme uniting "Pedipalpi" with Araneae was already mentioned (Fig. 3e). This finding was not surprising from the viewpoint of the systematists (who had known for a long time that these taxa are closely related because of other character states: Megoperculata) [137], but it was important to recognize that spermatology may function as a taxonomic tool in Arachnida [21, 76, 103, 107, 112]. However, the main problem with these taxa is concerned with the relationships within the group Megoperculata. Are Uropygi and Amblypygi sister groups and are they together (as Pedipalpi) the sister group of the taxon Araneae [117]? Are Amblypygi and Araneae sister groups (= Labellata) [137] forming the sister group to Uropygi? Does the term "Pedipalpi" thus describe a monophyletic or paraphyletic taxon [71]? Moreover, do the Uropygi really constitute a natural (monophyletic) group? Several authors have divided this taxon into Thelyphonida (=Uropygi *s. strict.*) and Schizomida (= micro whip scorpions) [90, 96, 97]? Are the Palpigradi closely related to the "Pedipalpi" (especially to Uropygi-Schizomida) as several authors have suggested [38, 83, 110, 132; see also 117]? Spermatology may at least point the direction of a solution of these questions (Fig. 5). In Araneae the nucleus of the sperm cell extends with a postcentriolar nuclear elongation beyond the base of the axoneme making the nucleus and the whole cell asymmetrical. In Amblypygi the same asymmetry of the nucleus is found, though less pronounced. Araneae and Amblypygi are thus united by the presence of this nuclear elongation as a synapomorphy (shared derived character). The presence of a middle piece in the Amblypygi and the early derived spider *Heptathela kimurai* (Mesothelae) is, in contrast, considered a symplesiomorphy. On the other hand Thelyphonida and Schizomida do not possess a middle piece and a shortened central triplet in the axoneme. Both characteristics are probable synapomorphies. The helical nucleus is not developed in a corkscrew (with sharpened edges) as in the Amblypygi and Araneae and does not demonstrate a postcentriolar nuclear elongation (symmetrical nucleus). These characteristics are most likely symplesiomorphies of the Uropygi. Thus the spermatological results would support the view that the taxon "Pedipalpi" describes indeed a paraphyletic group. There are no spermatological indications which would support a close relationship with Palpigradi. Schizomida possess the

FIG. 8. — Some characteristics of acarine sperm cells. **a:** Vacuolated-type sperm in *Sejus togatus* (Gamasida-Sejina) in transverse section. Note the wall of the vacuole provided with numerous so called cellular processes (compare c) [11]. x 14 600. **b:** Ribbon-type sperm of *Parasitus berlesei* (Gamasida-Parasitina) sectioned transversely. Note ribbons (arrowheads) with the paired sacs underneath [5]. x 16 500. **c:** Cellular processes in the spermatozoon of *Sejus togatus* (Gamasida-Sejina). Note different profiles according to level of section through the individual processes and filaments within the processes. Large arrowheads indicate microtubules, small arrowheads indicate flat acrosomal cisterna (compare e). x 84 000. **d:** Spermatozoon of *Cyta latirostris* (Actinedida). This type of sperm may be regarded as representative (plesiomorphic) of at least a great part of the very diverse Actinedida (see Fig. 7). Arrowheads indicate coils of the acrosomal filament [6]. x 18 500. **e:** Part of early spermatid of *Epicrius mollis* (Gamasida-Epicriina), which will develop into a vacuolated-type sperm. The acrosomal vacuole has differentiated into an acrosomal plate, to which the nucleus is attached and from which the acrosomal filament arises (arrow), and an extensive flat portion (cisterna) which grows out under the plasmalemma (arrowheads; compare c) [5]. x 27 000. APL, acrosomal plate; AV, acrosomal vacuole; N, nucleus; V, vacuole.

Source : MNHN, Paris



Source : MNHN, Paris

typical 9x2+3 axoneme of "Pedipalpi"-Araneae (synapomorphy of the Megoperculata), Palpigradi have aflagellate spermatozoa (Figs 2, 5). Further it appears that it is not necessary to separate Schizomida from the remaining Uropygi because of the mentioned synapomorphies, despite differences which include the paracrosomal lattice structure appearing in the late spermatid of *Schizomus* as a transient structure (Figs 3d, 5). The position of the acrosomal filament and the deep implantation fossa are most remarkable (Fig. 5) [21, 24, 76, 107, 126].

Within the Araneae it was shown that spermatozoa can be transferred to the female as numerous individual cells, each surrounded by its own sheath (cleistospermia), as numerous aggregates of individual cells, surrounded by a common sheath (coenospermia), as one tubular "spermatophore" (only in Telemidae) or as numerous syncytial spermatozoa (synspermia) (see above).

Though only few taxa have been studied with respect to these transport-forms it appears that the coenospermia are plesiomorphic since these have been observed in the early derived liphistiomorph spiders [HAUPT & KOVOOR, 1988 pers. comm.; ALBERTI, HAUPT & SCHWENDINGER, unpublished]. Furthermore, representatives of several families of orthognath spiders (Atypidae, Antrodiaetidae, Nemesiidae, Dipluridae, Theraphosidae) and of the labidognath spider family Filistatidae [24, 26, ALBERTI & COYLE unpublished, ALBERTI, HAUPT & SCHWENDINGER, unpublished] possess this type of encapsulated sperm. The "spermatophore" of Telemidae is regarded by the present author as an autapomorphy of this group of cave dwelling spiders since it has been observed only in this family (thus representing a rather isolated phenomenon within spiders) and does not show any relationship with spermatophores of Amblypygi, the assumed sister group of spiders. It is formed not by accessory glands, which are usually involved in spermatophore construction, but by secretions of the vasa deferentia [82]. Thus these "spermatophores" are established in the same region of the genital tract as the coenospermia and are most likely a special modification of these [12, 15, 24].

Synspermia are obviously an apomorphy. This type of sperm aggregation is not found elsewhere within the Arachnida and probably the animal kingdom. Until now, it has been observed only in the families Segestriidae, Dysderidae, Scytodidae and Sicariidae. It therefore may represent a synapomorphy uniting these (and other?) families of so-called haplogyne spiders, a group of Araneae which offers many systematical problems [46, 115] (Figs 3e, 6b).

The functional significance of the astonishingly diverse spermatozoa of Araneae, regarding the general uniform mode of insemination with the male palpal organ, has yet to be fully understood (see also Conclusions). However, the knowledge of spermatology is developed further in this functional respect and also with regard to systematical interpretations in Acari [5, 6, 9, 11, 13, 17].

The Acari are most often considered to be related to the Opiliones and/or Ricinulei [see 69, 70, 91, 117, 136, 137 for detailed discussions]. Other candidates are Solifugae and Palpigradi [67, 69, 70, 149]. However, it has long been debated what comprise the Acari. Are the Acari polyphyletic, diphyletic or monophyletic [see 129]? Aside of some extreme views which for example regarded the Opilioacarida or the Ixodida as independent arachnid groups or at least far separate from the (other) Acari [see 111, 116, 123], in the last decades two suggestions have mainly been discussed. Some authors support the idea that there are three (Opilioacarida, Anactinotrichida, Actinotrichida) major acarine groups [68, 79, 83] others think of two (Anactinotrichida including Opilioacarida, Actinotrichida) [53, 55, 69, 70, 85, 91, 97, 150]. Spermatology has contributed to these questions the following: from the sperm morphology of Opilioacarida, which is almost identical with that of Ixodida (ticks) and certain Gamasida, it is obvious that these taxa constitute one group: Anactinotrichida (=Parasitiformes). The vacuolated type of sperm is a synapomorphy of this group (Fig. 7). On the other hand it was demonstrated that the spermatozoa of Actinotrichida (=Acariformes) do not have anything in common with

Source : MNHN, Paris

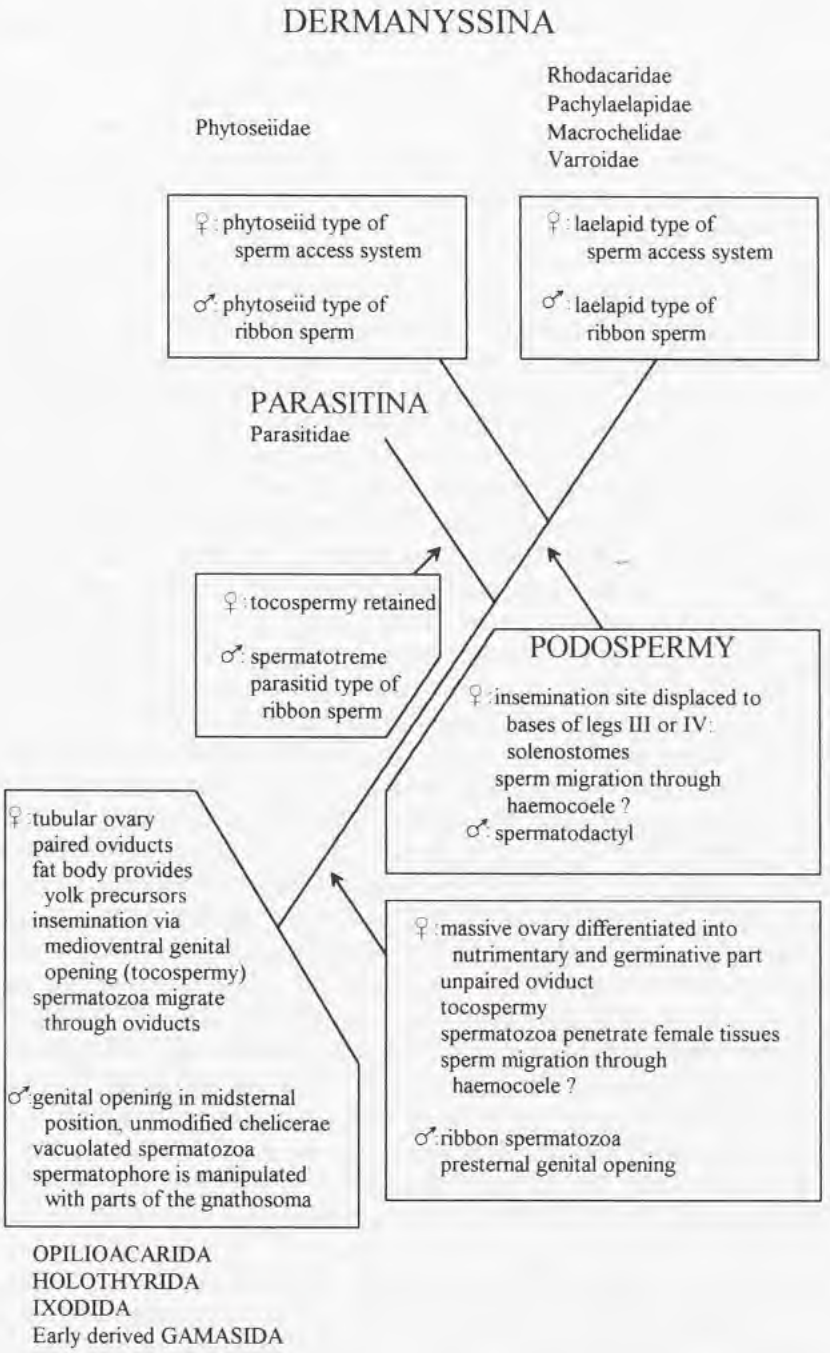


FIG. 9. — Diagram depicting probable evolutionary changes of genital systems and sperm morphology in Gamasida [13]. Only those gamasid families from which fine structural results are available are listed. Tocospermy: insemination via the primary genital opening; podospermy: insemination via secondary copulation pores (= solenostomes). Spermatotreme: slit in the mobile digit of the chelicera to hold the neck of the spermatophore during its transfer; spermatodactyl: slender process at the mobile digit of the chelicera used to transfer the sperm.

Source : MNHN, Paris

those of Anactinotrichida what could be termed a synapomorphy [5, 6, 9, 13]. Thus, it is at least a misleading simplification to consider the sperm cells of, for instance, Ixodida, as typical for the Acari as a whole (Fig. 7).

The problem of systematics of major groups of Acari is reduced by spermatological data to diphyly versus monophyly, with two different sperm types to be compared with each other and with those of the candidates outside the Acari (e.g. Ricinulei and Opiliones-Anactinotrichida; Solifugae and Palpigradi-Actinotrichida). No decision solving these questions for sister group relationships can yet be made (compare Fig. 2). The evolutionary distance between these taxa is probably too large [see for details 13]. At present the spermatological data also do not indicate that one of the major mite groups developed from part of the other, which would then be a paraphyletic taxon [see 30, 88].

Comparing sperm cells of Acari on a broad basis of taxa it was possible first to detect the profound difference between spermatozoa of Anactinotrichida and Actinotrichida (Fig. 7). Further, within the Anactinotrichida the vacuolated type turned out to be the plesiomorphic type from which other types (ribbon type and its subtypes) have developed [5, 6, 9, 13, 17, 139-141]. From this basis it was, at least in part, possible to trace the evolution of the sperm cell within the Anactinotrichida (Fig. 9). It was deduced that the vacuolated and ribbon type are correlated with different modes of insemination. In those taxa possessing vacuolated spermatozoa (Ixodida, Gamasida in part) sac like spermatophores are transferred to the female (reproductive behaviour of Opilioacarida and Holothyrida is not known). The gnathosoma manipulates the very complex spermatophore [53, 61] to the female genital opening located medioventrally. Capacitated spermatozoa (see above) migrate through the oviducts into the tubular ovary where fertilization of oocytes, most likely, occurs [43]. In those taxa having the ribbon type (Gamasida in part: Parasitina and Dermanyssina), the ovary comprises a nutritive tissue probably derived from sister cells of the oocytes [3, 25]. The ovary is a solid organ. It is thus necessary that female tissues are penetrated by the ribbon spermatozoa during their migration to the oocytes. There is growing evidence that this penetration results in a migration of spermatozoa through the haemocoele in Parasitina to the ovary [11, 13]. In Dermanyssina specialized paired sperm induction pores are found in a peculiar lateral position at the bases of legs III or IV [11, 17, 31, 50, 54, 57]. The question is, how was it possible to change the fertilization site during evolution of these taxa with spermatozoa apparently extremely derived (vacuolated type) and adapted to special conditions of the female genital system. However, knowing the situation in Parasitina it is not so difficult to understand this dislocation of the site of sperm transfer. Only the penetration site had to be displaced. Spermatophores were already manipulated with the chelicerae (Parasitina and Dermanyssina males have transformed chelicerae for this purpose: spermatotrema, spermatodactyl) and spermatozoa, of the ribbon type, were already adapted to penetration and migration through female tissues. Further adaptations are found in Dermanyssina regarding a new sperm access system which leads the sperm cells from the induction pores (solenostomes) to the ovary, displacement of the male genital opening into a presternal position and reorganization of the female genital tract and these in turn result in alterations of spermatozoa of the ribbon type to a less complex structure compared to the vacuolated type or the ribbon type of the Parasitina [11, 13, 17].

These interpretations which are based primarily on spermatological results, combined with studies on anatomy, histology and oogenesis, may very well play a key role in understanding the large group of Anactinotrichida and its most successful (species-rich) taxa Parasitina and Dermanyssina (Fig. 9). Evidently the modifications can be interpreted in the sense of economy, with the development of a nutritive tissue (so called lyrate organ) [95] in Parasitina and Dermanyssina as the initial event [11, 13, 17, 25]. It is of interest that comparable differentiations occurred in the Acari/Actinotrichida within the spider mites (Tetranychidae: direct sperm transfer with penis, migration of spermatozoa through haemocoele, nutritive cells in the ovary) and

Source : MNHN, Paris

Acaridida (penis, secondary copulatory pore, sperm access system leading to ovary) by convergence [13, 16, 22, 98, 145].

Thus the case of the Acari not only demonstrates the usefulness of comparative spermatology in solving systematical problems. It also gives an impression how sperm diversity has been evolved parallel to phylogenetic alterations with their functional/adaptive implications in a restricted taxon.

Conclusion and perspective

Though in recent years knowledge on sperm structure in Chelicerata has increased considerably, many questions remain unsolved. Indeed, the growth in knowledge has shown that yet more fascinating problems remain to be investigated only some of which can be indicated here.

First, spermatozoa of several taxonomic groups are only known from one (Solifugae, Ricinulei, Palpigradi) or very few species (e.g. Uropygi, Amblypygi) and it is evidently necessary to examine further representatives. From Solifugae only mature spermatozoa are known and studies on spermatogenesis are urgently required to understand the very peculiar sperm morphology as well as the aggregations which they form (Fig. 6d).

Capacitation processes are only known in (structural) detail from tick spermatozoa and similar events (e.g. the turning inside out-process, only to mention the most striking event) probably occur also in the vacuolated sperm of the other taxa. But there are indications of capacitation of sperm of other acarine taxa (e.g. Anactinotrichida: *Varroa jacobsoni*; Actinotrichida: *Tetranychus urticae, Bdella septentrionalis, Phytoptus avellanae, Acarus siro and Tyrophagus putrescentiae*) [5, 17, 19, 22, 23, 144].

In spiders, the synspermia of Dysderidae and also the cleistospermia of Oonopidae possess large vacuoles [see above, 24]. Probably these facilitate the process of uncoiling in the female. The organelles such as acrosomal vacuole, nucleus and axoneme, are covered with membranes of the vesicles/vacuole, which could be integrated into the plasmalemma very quickly. Thus these spermatozoa may be regarded as "precapacitated" (being somewhat similar by convergence to the vacuolate sperm cells of the mentioned Acari-Anactinotrichida) (see also vesicles at the periphery of sperm of Uropygi and Amblypygi; Fig. 2). As the female genital tract of spiders differs considerably [65], these and other specializations (e.g. the lack of mitochondria in some spiders) may represent adaptations to these various fertilization conditions. These problems evidently offer a broad field for exciting future studies which may be considered under the aspects of sperm competition and securing sperm priority.

Except for *Limulus polyphemus* sperm, nothing is known about acrosomal reactions in chelicerate sperm cells [124]. In most taxa the exact site where the eggs are fertilized is unknown or at least speculative. Evidently sperm cells pass through the haemolymphatic space in certain mites (Acari: Parasitidae, Tetranychidae). Are these exceptions or do further examples exist? Apparently this passage brought about the development of new genital ducts (sperm access system in Dermanyssina) as a reaction of the female comparable to the paragenitalia in bed bugs and their relatives. This in turn indicates the strong selective influence which is imposed by the females not only on the male genitalia but also on spermatozoa [see e.g. 11, 13, 17, 51].

Only few chelicerate sperm cells have been investigated with regard to their cytochemistry: *Limulus polyphemus* [124] and two gamasid mites [143]. The significance of the many enigmatic components (vacuoles, vesicles, dense streaks, plates, various filaments, inclusion bodies, etc.) could be better understood using appropriate and available methods.

Even in those groups in which a broad range of taxa has been investigated, such as Araneae and Acari, much work is still to be done. Araneae offer many questions, for instance: what is the functional significance of the various transport-forms (coenospermia, cleistospermia, synspermia, "spermatophores")? Is this diversity related to the male and female copulatory organs which are

Source : MNHN, Paris

insufficiently understood [see 65, 73, 87, 121, 122, 128]. How are these encapsulated spermatozoa activated and how do they then function? What happens to the enigmatic synspermia in the female?

Sperm aggregates of Solifugae and the enigmatic small opilionids of the genus *Siro* need further investigation. The latter taxon is the only one within the Chelicerata known to have dimorphic spermatozoa, albeit of unknown functional significance. Does this phenomenon also occur in other Cyphophthalmi?

The peculiar genital tract of "Pedipalpi", possessing a pair of glands with holocrine secretion of a very unconventional type, offers very exciting interpretations. If these glands could be proven to be of testicular origin, the secretory products could represent strongly modified germ cells too. This interpretation would be of much interest regarding the relationships between "Pedipalpi" and Araneae [see above and 13].

The adaptations of sperm cells being transferred via various types of secreted containers (capsules, stalked spermatophores of various complexity) [see e.g. with regard to Acari: 27, 61, 64, 146] need to be studied in more detail.

Only few studies have focused on the association of microorganisms with spermatozoa [2, 59, 62]. In particular the enigmatic *Adlerocystis* sp. which is a regular symbiotic associate of tick spermatozoa deserves more attention [62].

In Acari a broad range of sperm cells from a very high complexity to very "simple" cells is observable (Figs 7, 8) All these cells transfer genetic material apparently successfully. Many taxa, however, reproduce exclusively parthenogenetically [100]. This is in particular remarkable regarding the oribatid mites, a very common group living in almost all soils. It has been suggested that in certain taxa a reversion to sexuality may have occurred [99]. An even more striking example is represented by the Acaridida (flour mites, house dust mites, feather mites, itch and mange mites etc.), which reproduce bisexually and have probably developed from within a certain group of oribatids which is known to reproduce exclusively by parthenogenesis. The idea has been suggested (as the most parsimonious one) that the Acaridida developed bisexuality, and thus sperm cells, secondarily [99, 100].

Mites demonstrating the most diverse reproductive behaviour are probably one of the most promising groups to study the complex interrelationships between behavioural, morphological and cytological evolutionary processes on a comparative basis in Chelicerata. Such a study almost certainly would give further material for improving systematical concepts.

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Source : MNHN, Paris

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Source : MNHN, Paris

Spermatozoal Ultrastructure in Dendrobranchiata (Crustacea, Decapoda): Taxonomic and Phylogenetic Considerations

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ABSTRACT

This chapter reviews the diverse spermatozoal patterns found to date in Dendrobranchiata, including the first ultrastructural descriptions of solenocerid and sergestid spermatozoa. Some characters are analysed from a spermiocladistic perspective and a tentative phylogram is presented where the dendrobranchiate sperm forms are related to phyletic arrangements inferred from holomorphological studies. Evolutionary relationships within Eucarida are discussed with respect to the various sperm morphologies.

RÉSUMÉ

Ultrastructure du spermatozoïde chez les Dendrobranchiata (Crustacea, Decapoda): considérations taxonomiques et phylogénétiques

Ce chapitre synthétise nos connaissances sur les différentes morphologies des spermatozoïdes rencontrées jusqu'ici chez les Dendrobranchiata, y compris les premières descriptions ultrastructurales du spermatozoïde d'un Solenoceridae et d'un Sergestidae. Quelques caractères sont analysés dans une perspective spermiocladistique et un essai de phylogramme est présenté, dans lequel les morphologies des spermatozoïdes des Dendrobranchiata sont mises en relation avec les arrangements phylogéniques issus des études holomorphologiques. Les relations évolutives chez les Eucarides sont discutées en relation avec les différentes morphologies des spermatozoïdes.

The almost limitless morphological diversity of animal spermatozoa has inspired an extensive amount of research work in diverse biological fields. Specifically, in recent years sperm ultrastructure has become a useful tool in studies on taxonomy and phylogeny, since it proves effective in resolving problems which escape conventional somatic analyses [25, 30]. The shape and inner organization of a given sperm cell is definitely characteristic of the species that produces it; that is, the spermatozoal ultrastructure constitutes a distinctive character of identity for every animal species. Correspondingly, it is obvious that the evolution of a spermatozoon runs parallel to the evolution of the corresponding species from which it comes. On this basis, an increasing number of works have been conducted to establish a congruent relationship between the sperm

MEDINA, A., 1995. — Spermatozoal ultrastructure in Dendrobranchiata (Crustacea, Decapoda): taxonomic and phylogenetic considerations. *In*: JAMIESON, B. G. M., AUSIO, J., & JUSTINE, J.-L. (eds), Advances in Spermatozoal Phylogeny and Taxonomy. *Mém. Mus. natn. Hist. nat.*, **166** : 231-242. Paris ISBN : 2-85653-225-X.

Source : MNHN, Paris

ultrastructure and the evolution and phylogeny of many animal taxa. JAMIESON [25] coined the terms "spermiocladistics" and "spermiotaxonomy" in reference to the application of sperm ultrastructure to phylogenetic and taxonomic studies.

The astonishing diversity of forms that animal spermatozoa can adopt is well exemplified by the crustaceans, where a wide range of sperm types vary from the aquasperm-like (plesiomorphic) remipedian spermatozoon, through the amoeboid and acrosome-less forms present in several taxa, to the aflagellate, either "unistellate" or "multistellate", decapod sperm [31]. The extreme strangeness of these spermatozoa aroused the curiosity of early spermatologists. Light microscopical reports on crustacean spermatozoa are relatively abundant and precocious, some of them including phylogenetic analyses based on the sperm types of decapods (see review by FELGENHAUER & ABELE [19]). With the development of electron microscopy, the opportunity to examine in detail subcellular structures opened the range of possibilities to utilize sperm morphology in reconstruction of crustacean phylogeny. Three recent reviews have painstakingly updated the knowledge on the sperm ultrastructure in decapods [19, 31, 39]. These revisions report the traditional tendency to classify the different decapod sperm morphologies into two categories, following early classifications of the Decapoda into Natantia and Reptantia. Thus, a supposedly uniform sperm plan, referred to as the "unistellate spermatozoon", was thought to be shared by the suborder Dendrobranchiata and the pleocyematan infraorder Caridea (formerly grouped in the Natantia along with the Stenopodidea), the "multistellate spermatozoon" being typical of the rest of the representatives of the suborder Pleocyemata (former Reptantia) [6]. Most recent studies, however, have provided additional information which recommends reconsideration of this view. Actually, the general unistellate sperm pattern appears to encompass two significantly distinct sperm structures [44]. Furthermore, other sperm morphologies present in species of Dendrobranchiata do not fit into either of the two traditional categories of decapod spermatozoa ([13], MEDINA, other chapter in this book [45]).

TABLE 1. — List of Dendrobranchiata for which sperm ultrastructure is known

Family	Species	Reference
Penaeidae	*Parapeneus longirostris* (Lucas, 1846)	[44]
	Peneopsis serrata Bate, 1881	[47]
	Penaeus aztecus Ives, 1891	[9]
	Penaeus japonicus Bate, 1888	[48, 50]
	Penaeus kerathurus (Forskål, 1775)	[48]
	Penaeus setiferus (Linnaeus, 1767)	[18, 39]
	Penaeus vannamei Boone, 1931	[15, 39]
	Trachypeneus similis (Smith, 1885)	[39]
Sicyonidae	*Sicyonia brevirostris* Stimpson, 1874	[6]
	Sicyonia carinata (Brünnich, 1768)	[47]
	Sicyonia ingentis (Burkenroad, 1938)	[37, 60]
Aristeidae	*Aristaeomorpha foliacea* (Risso, 1827)	[45]
	Aristeus antennatus (Risso, 1816)	[13, 14, 45]
Solenoceridae	*Solenocera membranacea* (Risso, 1816)	[This study]
Sergestidae	*Sergestes arcticus* Kroyer, 1855	[This study]

Source : MNHN, Paris

Spermatozoal ultrastructure is well known for numerous species of reptant crustaceans, notably brachyurans. In a series of exemplary works, JAMIESON and co-workers [22, 27-29, 32-34] (see also JAMIESON, this volume) have applied spermiocladistics in this group with considerable success. However, the amount of information available on spermatozoal ultrastructure in Dendrobranchiata is more limited and mostly deals with the genera *Penaeus* and *Sicyonia* (Table 1). Wide gaps in the current knowledge of the ultrastructure of spermatozoa in Dendrobranchiata render it impracticable to perform a complete parsimony analysis of the various families and the phylogenetic relationships between them and with other decapod taxa. Nevertheless, spermiocladistic criteria may well be applied to phylogeny and taxonomy of the suborder. This chapter summarizes pre-existing information and contributes original observations, tentatively aiming at establishing valid foundations for spermiotaxonomy and spermiocladistics in the Dendrobranchiata that may be useful in forthcoming studies on this crustacean taxon.

Dendrobranchiate spermatozoal patterns

Thus far, ultrastructural descriptions of spermatozoal morphologies are available for representatives of three of the four families of the superfamily Penaeoidea: Penaeidae, Sicyonidae and Aristeidae (see Table 1 for list of species investigated). This previous information is here augmented with observations on the sperm of *Solenocera membranacea* as a representative of the family Solenoceridae. A brief ultrastructural description of the sperm of *Sergestes arcticus* is also provided in order to include a member of the superfamily Sergestoidea in support of general phylogenetic considerations. In aspects of systematics, I will here follow the classification proposed by BOWMAN & ABELE [5], whereas the spelling of taxa, and particularly that of names derived from *Pen(a)eus*, will be the one used and recommended by SCHRAM [59].

Family Penaeidae. Within the Dendrobranchiata, penaeid sperm have been the most extensively studied in terms of the number of species examined. These include five *Penaeus* species (*P. setiferus*, *P. vannamei*, *P. aztecus*, *P. japonicus* and *P. kerathurus*), *Trachypeneus similis*, *Parapeneus longirostris* and *Peneopsis serrata* (Table 1). In gross morphology, the peneid spermatozoon basically consists of a subspheroidal or ovoid main body and a spike. The main body comprises the central nuclear region, a cytoplasmic band surrounding it posterolaterally, and the acrosomal cap, which overlies the nuclear region anteriorly and is prolonged into a tapering spike (Fig. 1a, b). Both spike and acrosomal cap make up a membrane-bound acrosomal vesicle, with heterogeneous contents, which is directly invested by the plasma membrane. In particular, the spike morphology and substructure vary markedly from species to species. The whole acrosomal complex is completed with the subacrosomal region, which is quite simple in this family, merely containing a sparse flocculent material between the chromatin and acrosomal cap.

The sperm of *Parapeneus longirostris* and *Peneopsis serrata* have a central protuberance at the concave side of the acrosomal cap immediately opposite the spike (Figs 1b, 2h, i). This supposed synapomorphy is consistent with the close phylogenetic proximity of the genera *Parapeneus* and *Peneopsis*, both grouped together by BURKENROAD [8] within the tribe Parapeneini, which also includes *Artemesia* and *Metapeneopsis*. Confirmation of such a structure in the latter genera would strengthen phylogenetic unity of this taxon.

As in all dendrobranchiate species whose spermatozoon has been ultrastructurally studied, the nuclear region of peneid sperm consists of a non-membrane-bound, filamentous chromatin mass. Posterolaterally, the chromatin is surrounded by a band of cytoplasm which contains membrane lamellae, vesicles and mitochondria-like bodies, but lacks centrioles and microtubules. Within the Dendrobranchiata, the sperm of *Penaeus japonicus* are exceptional in that they exhibit several microtubule bundles in the cytoplasm [48]. The microtubules appear in primary spermatocytes of *P. japonicus* and are retained through spermiogenesis to the mature

Source : MNHN, Paris

spermatozoon (personal observation). In other peneid species (e.g. *Penaeus kerathurus*, *Parapeneus longirostris*), microtubules are absent from all spermatogenetic stages.

Recent molecular studies [51] have revealed extensive genetic differences between species of *Penaeus* which have not been accompanied by substantial evolutionary morphological changes. This is congruent with the occurrence of diverse species-specific dissimilarities leading to different ultrastructure of sperm in the genus *Penaeus* and in general in the Penaeidae, and confirms the taxonomic validity of the sperm morphology in the Dendrobranchiata.

Family Sicyonidae. Ultrastructural data have been reported for three *Sicyonia* species: *S. brevirostris*, *S. carinata* and *S. ingentis* (Table 1). The inner morphological organization of the spermatozoon is very similar in *S. ingentis* [37] and *S. carinata* [47]. In general, as in Penaeidae, the sperm consist of an acrosomal vesicle (formed by the spike and acrosomal cap), subacrosomal region, and nuclear region surrounded by a cytoplasmic band (Fig. 1c). Anteriorly, the acrosome and plasma membranes are closely joined. As a taxonomically significant difference, the spike of *S. ingentis* is spiralled, whereas that of *S. carinata* is smooth. The plesiomorphies (1) absence of nuclear envelope and (2) perinuclear cytoplasmic band (containing small and large vesicles and lacking microtubules) are also found in this sperm type. Nevertheless, the highly elaborate subacrosomal region (comprising diverse distinct structures) [37, 47] appear to be a clear autapomorphy of the family Sicyonidae. Compared to the spermatozoa of the other dendrobranchiate families, the acrosomal vesicle shows the apomorphic character that the posterior membrane of the acrosomal cap is intricately folded in a ring of convoluted membrane pouches or digitations [37, 47] (Fig. 1c).

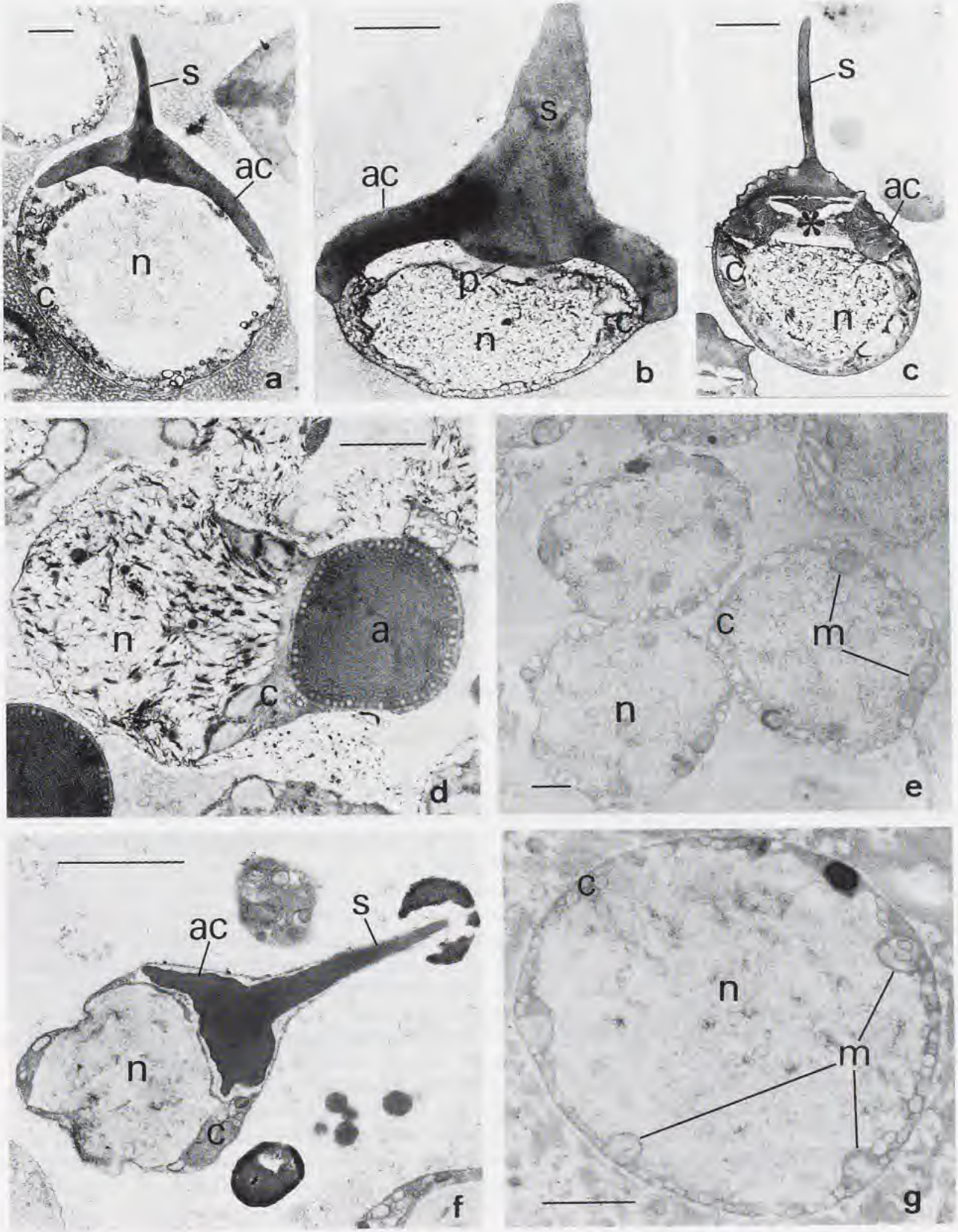
In a long series of valuable works, CLARK and co-workers have described morphological details of the acrosome reaction in *S. ingentis* [10-12, 20, 21, 24, 62]. These accounts reveal the role played by each of the spermatozoal components during fertilization, hence they greatly aid understanding of the biological significance of the acrosomal structures in dendrobranchiates.

Family Aristeidae. The relatively high ultrastructural homogeneity found within the Penaeidae and Sicyonidae is not seen in the Aristeidae. Studies of *Aristeus antennatus* [13, 14, 45] and *Aristaeomorpha foliacea* [45] indicate the existence in the family of at least two different ultrastructural sperm plans that are in turn discordant with the peneid-sicyoniid assemblage. The *A. antennatus* (Figs 1d, 2c) sperm type exhibits diverse peculiarities in comparison with the other Dendrobranchiata. First, its spherical acrosome does not cap the nuclear region and lacks both spike and subacrosomal region; the inner arrangement of the acrosomal contents is complex and different from that of any other known dendrobranchiate spermatozoon. Secondly, the cytoplasm does not constitute a band around the filamentous chromatin mass, but is accumulated in a collar between the acrosome and nuclear region, enclosing mitochondria-like vesicles and membrane lamellae. Consequently, most of the chromatin is bounded directly by the plasma membrane, since the nuclear region is, as in all dendrobranchiates, not membrane-bound. I agree with DEMESTRE & FORTUÑO [13] that the basic sperm structure of *A. antennatus* resembles that of spiny lobsters, *Panulirus* spp. [61], although with the highly significant absence of the typically reptantian radial arms, which suggests parallelism rather than a close phylogenetic relationship.

The *Aristaeomorpha foliacea* sperm type (Figs 1e, 2b), lacking the acrosome, also differs from the dendrobranchiate unistellate spermatozoal morphology. It consists of a central nuclear region entirely surrounded by the plesiomorphic cytoplasmic band, which includes membrane lamellae, small peripheral vesicles and mitochondria-like bodies. Plesiomorphic features are also the absences of nuclear envelope, centrioles and microtubules.

FIG. 1. — Transmission electron micrographs of spermatozoa. **a:** *Penaeus japonicus*; **b:** *Parapeneus longirostris*; **c:** *Sicyonia carinata*; **d:** *Aristeus antennatus*; **e:** *Aristaeomorpha foliacea*; **f:** *Solenocera membranacea*; **g:** *Sergestes arcticus*. Scale bars: 1 µm. a: acrosome, ac: acrosomal cap, c: cytoplasm, m: mitochondria-like bodies, n: nuclear region, p: protuberance of the acrosomal cap, s: spike, *: subacrosomal region.

Source : MNHN, Paris



Source : MNHN, Paris

Family Solenoceridae. The spermatozoon of *Solenocera membranacea* is similar to that of Penaeidae in general morphology (Figs 1f, 2d), though it shows conspicuous differences with regard to the other spiked dendrobranchiate sperm. The contents of the acrosomal vesicle are homogeneously electron-dense and the cap appears asymmetrical in sagittal sections, one of its lateral expansions projecting further than the other. Another distinctive feature of the *S. membranacea* spermatozoon is that the plasma membrane becomes separated from the anterior acrosome membrane, the intervening space being occupied by part of the cytoplasmic mass. The perinuclear cytoplasm is rather amorphous, though parallel lamellae and mitochondria-like bodies may be recognized. It is thick under the lateral edges of the acrosomal cap and grows thinner at the posterior part of the sperm. Anteriorly, it forms a thin band separating the scarce subacrosomal substance from the finely filamentous chromatin, a feature that recalls that observed in the penaeid *Parapeneus longirostris* [44].

Family Sergestidae. The sperm of *Sergestes arcticus* are simple, spheroidal or slightly ellipsoidal cells which much resemble those found in *Aristaeomorpha foliacea*. They consist of a central, non-membrane bound nuclear region and surrounding cytoplasm (Figs 1g, 2a). The finely filamentous chromatin mass is encircled by a thin cytoplasmic band that mainly contains densely-packed electron-clear vesicles and a few mitochondria-like bodies. Occasionally, the cytoplasm encloses lipid-like, highly osmiophilic inclusions. At some points, the cytoplasmic band may be interrupted, thus allowing a direct contact of the nucleoplasm with the plasma membrane. Acrosome, microtubules and centrioles are absent.

In eucarids absence of the acrosome had been reported only in Euphausiacea [31] and Stenopodidea [19]. Indeed, there appear to be striking resemblances between the spermatozoa of *Sergestes arcticus*, *Aristaeomorpha foliacea* (Fig. 1e, g) and *Euphausia* sp. (see [31]) which very probably are indicative of phylogenetic relationship. These are: (1) central nuclear region consisting of diffuse, finely filamentous chromatin, (2) complete disruption of the nuclear envelope, (3) vesiculate, thin perinuclear cytoplasmic band, (4) absence of centrioles and microtubules, and (5) absence of acrosome. Now the question arises as to the evolutionary meaning of acrosome-less spermatozoa within Dendrobranchiata. Has this condition been acquired secondarily or is it a primitive one? This subject is discussed below.

Sperm phylogenetic relationships within Dendrobranchiata and between Dendrobranchiata and other Eucarida

The present survey suggests as clear dendrobranchiate spermatozoal symplesiomorphies: (1) complete loss of the nuclear envelope, (2) filamentous chromatin, (3) absence of centrioles, (4) absence of radial (stellate) arms. The plesiomorphic perinuclear distribution of the cytoplasm does not occur in *Aristeus antennatus*; in this species, the cytoplasm forms a collar between the acrosome and nuclear region. Whether the acrosome-less condition of *Aristaeomorpha foliacea* is an apomorphic character or, in contrast, a plesiomorphy, is a matter that remains to be established when more data are available. Nonetheless, the finding of similar, acrosome-less sperm patterns in euphausiids (*Euphausia* sp.) [31], stenopids (*Stenopus hispidus*) [19], sergestids (*Sergestes arcticus*) and aristeids (*Aristaeomorpha foliacea*) appears to point to its plesiomorphy. Although the loss of the acrosome is a repeated event throughout evolution of the crustacean sperm [26], the assumption of sperm originally endowed with an acrosome would suppose the highly improbable independent loss of the acrosome in several separate lineages of the eucarid tree (Fig. 2).

According to JAMIESON [31], "the malacostracan acrosome is a new development, in view of evidence that their acrosome originates from the endoplasmic reticulum and not, as is usual, from the Golgi." Certainly, several studies have demonstrated that the acrosomal structures in Decapoda derive from, or in association with, cisternae of the endoplasmic reticulum itself or of its specialized portion constituting the nuclear envelope [1-3, 14, 23, 35, 38, 40, 43, 44, 46, 49, 53, 55, 56, 58, 60]. Consequently, it can be said that the mechanisms involved in differentiation

Source : MNHN, Paris

of the acrosomal structures are somehow plesiomorphic. It is not known whether similar mechanisms take place during spermiogenesis in euphausiids, sergestids, stenopids and acrosome-less aristeids (as they do in fact in *Aristeus antennatus*). If so, it is to be assumed that the capacity to build acrosomes with the involvement of endoplasmic reticulum membrane systems (irrespective of the appearance or not of a distinct acrosome in mature sperm) was present in ancestors of eucarids before separation of euphausiids and decapods. Such a widely shared mechanism of acrosome formation is consistent with the occurrence of apparent homologies in the acrosomes of as distant taxa as the Penaeidae and the Brachyura [46]. In contrast, the caridean spike, in spite of resulting in a sperm pattern closely resembling the peneid-sicyoniid-solenocerid one, is not to be phylogenetically related to the dendrobranchiate spike [44]. Definitely, comparative sperm ultrastructural studies argue against a monophyletic "Natantia", as BURKENROAD [8] conjectured more than a decade ago.

If, as appears plausible (see above), the absence of an acrosome is plesiomorphic, then the spiked acrosome of dendrobranchiates would be a synapomorphy of the families Penaeidae, Sicyonidae and Solenoceridae, whereas the sperm of the Sergestidae and Aristeidae should be considered as more primitive, that of *Aristeus antennatus* showing secondary (thus apomorphic) acquisition of the acrosome independent of the evolutionary line leading to the other acrosome-possessing dendrobranchiate spermatozoa. Taking into account the report, albeit requiring confirmation, that euphausiid spermatozoa [31] are similar to those of *Sergestes arcticus* and *Aristaeomorpha foliacea* (Figs 1e, g, 2a, b), occurrence of a plesiomorphic acrosome-less sperm is congruent with the statement of BURKENROAD [8] that the ancestors of the Decapoda were more euphausiid-like than the modern forms. According to this, the primitive eucarids could have euphausiid-like sperm, euphausiids, sergestids, aristeids and stenopids having retained this pattern. Among Aristeidae, some representatives (*A. antennatus*) might well have recreated a spheroidal acrosome with no ultrastructural resemblance to the acrosome of any of the other known dendrobranchiates, the sperm becoming arranged into a reptant-like pattern (although retaining the plesiomorphic absences of arms, microtubules and nuclear envelope, and therefore with no apparent direct phylogenetic relation to reptants) which represents an independent evolutionary line (see Fig. 2).

Spermiocladistic support for the statement of FELGENHAUER & ABELE [17] that the Caridea and Stenopodidea derive from ancestral reptants would necessitate further research. Derivation of carids from primitive thalassinoids is not congruent with most recent observations on spermatozoal ultrastructure by TUDGE [this volume], unless important deviations (= apomorphies) from the reptant ground plan be assumed, namely the loss of the membrane-bound acrosome and of microtubule-containing radial arms, as well as the independent development of a non-membrane bound spike [2, 3, 16, 18, 38, 42, 52, 54, 56, 57] that acts in a distinct and very particular manner during fertilization [4, 41]. These typically caridean characteristics confirm a sperm pattern that represents a fairly distinct, clearly identifiable evolutionary trend within the Decapoda.

The occurrence of either a complete or a discontinuous double-membrane nuclear envelope, partially invested by the plasma membrane, as well as the occasional presence of centrioles at the base of the acrosome, are shared by carids and reptantians, these features supporting a certain unity of both groups. However, the supposed reptantian origins of stenopodideans [17] are disputed by the ellipsoidal, arm-less and acrosome-less form of the spermatozoon of *Stenopus hispidus* [19], which is also characterized by having a lamellar body located against the plasma membrane at one side of the sperm cell (a structure that strongly suggests reminiscence of the well developed membrane system associated with proacrosomal vesicles in decapods). At first glance, this sperm morphology would place the stenopodideans close to the euphausiids, hence suggesting an early separation of Stenopodidea from the reptantian-caridean stem just above the origin of the Dendrobranchiata and before appearance of the acrosome and of appendages in decapod spermatozoa (see Fig. 2).

Source : MNHN, Paris

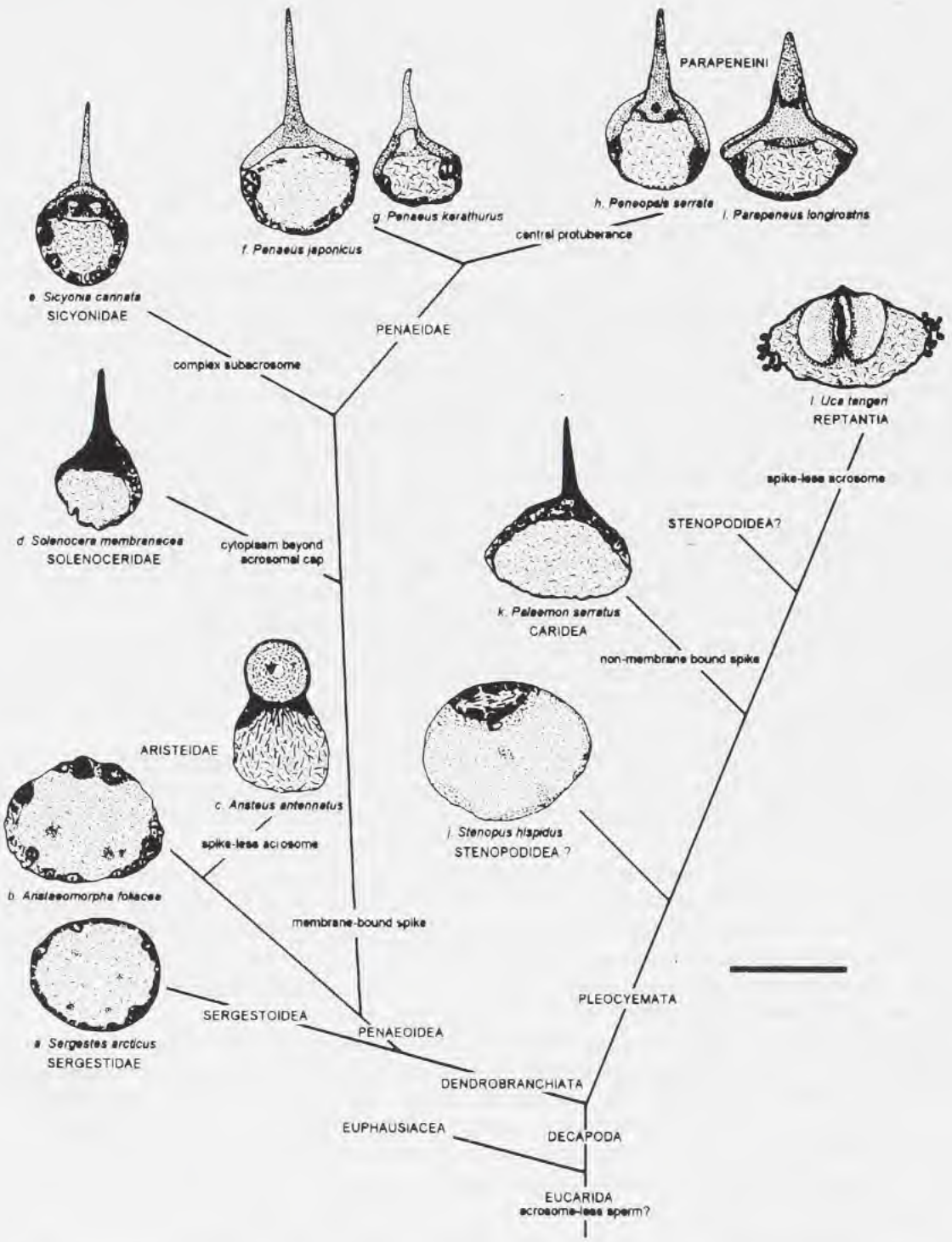


FIG. 2. — Intuitive phylogenetic tree of the sperm of the Eucarida where the different dendrobranchiate spermatozoal patterns are represented. Figures b, c, e-k have been prepared from drawings or micrographs which appeared in [19, 44-48, 52]; a and d are original. Scale bar: 8 µm for k, 4 µm for the others.

Source : MNHN, Paris

Figure 2 represents a tentative phylogenetic tree which attempts to reconcile the current knowledge of spermatozoal ultrastructure in eucarids with phyletic relationships suggested recently by reputable authors [7, 17, 26, 31, 36, 59]. Although the phylogram is necessarily provisional owing to the limited number of studies available, the most important spermatozoal evolutionary trends are represented in it. In the proposed sperm phylogram, separation of the Euphausiacea is followed by a node grouping the Decapoda, with two distinct evolutionary lines, one of which leads to Dendrobranchiata and the other to Pleocyemata. It is believed that the decapod sperm were originally devoid of an acrosome, a condition that was retained in Sergestidae as well as representatives of the family Aristeidae (*Aristaeomorpha foliacea*). However, another aristeid (*Aristeus antennatus*) has a spermatozoon supplied with an apomorphic membrane-bound acrosome that resembles the sperm of *Panulirus* spp. owing to concurrence of several parallelisms rather than to phylogenetically-based shared features. The three other families of the Dendrobranchiata have in common spermatozoa which share a synapomorphic membrane-bound acrosomal spike. From the node uniting these non-aristeid sperm, the first branch to emerge is represented by the spermatozoon of *Solenocera membranacea*, which shows an asymmetrical acrosomal cap and separation of the plasma and anterior acrosome membranes, allowing part of the cytoplasm to "leak" beyond the acrosomal cap. Finally, Sicyonidae and Penaeidae appear as two aligned groups, the sperm of which are easily distinguishable by the highly complicated, apomorphic subacrosomal region present in sicyoniids, in contrast to the simple one of penaeids. In the Penaeidae, two distinct sperm types have been recognized on the basis of the presence (*Parapeneus longirostris* and *Peneopsis serrata*) or absence (*Penaeus* spp.) of a central protuberance at the concave side of the acrosomal cap. This dendrobranchiate sperm phylogenetic arrangement is in agreement with the close interrelation that Burkenroad [8] suggests between penaeids and sicyoniids. However, with our limited information, no spermatozoal evidence has been found to ally, as he claims, aristeids and solenocerids. On the contrary, the spermatozoon of *S. membranacea* resembles the Penaeidae-Sicyonidae sperm rather than any of the known Aristeidae sperm types.

The Pleocyemata lineage (Fig. 2) would first include acrosome-less sperm forms, such as those present in *Stenopus hispidus*. Therefore, a logical phylogenetic sequence would suggest a first offshoot leading to Stenopodidea in a scheme that is congruent with the phylogram of FELGENHAUER & ABELE [17]. However, another spermatologically plausible, albeit less probable, arrangement following the more recent cladograms of SCHRAM [59] and KIM & ABELE [36], would place the offshoot of Stenopodidea between the branches leading to Caridea and Reptantia.

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Source : MNHN, Paris

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Source : MNHN, Paris

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Source : MNHN, Paris

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Source : MNHN, Paris

The Atypical Sperm Morphologies of *Aristeus antennatus* and *Aristaeomorpha foliacea* (Crustacea, Dendrobranchiata, Aristeidae) and Their Phylogenetic Significance

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ABSTRACT

The spermatozoa of *Aristeus antennatus* and *Aristaeomorpha foliacea* show two distinct morphological patterns which greatly differ from each other and are also distant from the common dendrobranchiate sperm plan. In *A. antennatus*, the spermatozoon possesses a conspicuous spherical acrosome which lacks both spike and subacrosomal region. Its multilayered inner structure is not comparable to the acrosomal arrangement of any other decapod, and is postulated to be the result of independent evolution. The cytoplasm is concentrated in a collar between the acrosome and the non-membrane bound nuclear material; therefore, it displays a subacrosomal (apomorphic) rather than perinuclear distribution. In spite of the plesiomorphic lack of radial arms, which is common to all dendrobranchiates, the general morphology of this spermatozoon resembles that of reptant decapods in several respects. The absence of a spike, the spherical (not capping) acrosome, and the subacrosomal (instead of perinuclear) cytoplasm are considered as apomorphies. The most significant feature of *Aristaeomorpha foliacea* spermatozoon is the absence of the acrosome. This simple sperm cell consists of the central nuclear material encompassed by a cytoplasmic band that is in turn bounded by the plasma membrane. Typical dendrobranchiate (plesiomorphic) features of *A. foliacea* sperm are the perinuclear cytoplasm (containing small peripheral vesicles, mitochondria and membrane lamellae), the non-membrane bound nuclear material, and the absences of centrioles and radial arms. The two spermatozoal patterns described hereafter appear to be the result of distinct evolutionary trends.

RÉSUMÉ

Les morphologies atypiques des spermatozoïdes d'*Aristeus antennatus* et *Aristaeomorpha foliacea* (Crustacea, Dendrobranchiata, Aristeidae) et leur signification phylogénétique.

Les spermatozoïdes de *Aristeus antennatus* et *Aristaeomorpha foliacea* possèdent des morphologies bien distinctes entre elles et différentes de la morphologie habituelle des Dendrobranchiata. Le spermatozoïde d'*A. antennatus* possède un important acrosome sphérique dépourvu d'épine et de région subacrosomienne. Sa structure interne à plusieurs couches n'est comparable à l'acrosome d'aucun autre Décapode, et on fait l'hypothèse qu'elle est le résultat d'une évolution indépendante. Le cytoplasme est concentré en un collier entre l'acrosome et le matériel nucléaire non limité par une membrane. De ce fait, il montre une distribution subacrosomienne (apomorphe) plutôt que périnucléaire. En dépit de l'absence, plésiomorphe, de bras radiaux, qui est commune à tous les Dendrobranchiata, la morphologie générale de ce spermatozoïde ressemble à celle des Décapodes Reptantia pour plusieurs aspects. L'absence d'épines, l'acrosome sphérique (pas en capuchon), et le cytoplasme subacrosomien (au lieu de périnucléaire) sont considérés comme des apomorphies. La

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Source : MNHN, Paris

caractéristique la plus significative du spermatozoïde d'*Aristaeomorpha foliacea* est l'absence d'acrosome. Ce spermatozoïde simple consiste en un matériel nucléaire central entouré par une bande cytoplasmique, elle-même limitée par la membrane plasmique. Les caractéristiques (plésiomorphes) de Dendrobranchiata du spermatozoïde d'*A. foliacea* sont un cytoplasme périnucléaire (contenant des petites vésicules périphériques, des mitochondries et des lamelles membranaires), le matériel nucléaire non limité par une membrane, et l'absence de centrioles et de bras radiaux. Les deux morphologies de spermatozoïde décrites ici semblent être le résultat de deux tendances évolutives distinctes.

The spermatozoa of dendrobranchiate shrimp are typically described as aflagellate cells consisting of a rounded main body partially encompassed by a cap with a protruding spike [5, 9, 11]. The most prominent feature in this sperm type is the presence of a single appendage, the so-called spike, which characterizes the "unistellate" condition of "natantian" spermatozoa, distinguishing it from the "multistellate" condition of "reptantian" sperm. However, the spike-less sperm morphologies of *Aristeus antennatus* and *Aristaeomorpha foliacea* provide exceptions to the supposed spermatozoal uniformity in the Dendrobranchiata and recommend a thorough analysis of the sperm ultrastructure of both species of Aristeidae. Though DEMESTRE & FORTUÑO [3] briefly described the spike-less spermatozoon of *A. antennatus* from scanning electron micrographs, an accurate characterization requires further investigation by means of transmission electron microscopy. Phylogenetic implications of both dendrobranchiate sperm morphologies will be considered in the context of the decapod sperm configuration.

MATERIALS AND METHODS

Specimens of *Aristeus antennatus* (Risso, 1816) and *Aristaeomorpha foliacea* (Risso, 1827) were collected off the Gulf of Cádiz in October 1993. Small fragments of vasa deferentia including ampullae were fixed in 2.5% glutaraldehyde in filtered seawater for 4-12 h, postfixed in 1% osmium tetroxide and dehydrated through acetones. For transmission electron microscopy, the samples were then infiltrated and embedded in ERL [22]. Ultrathin sections were viewed in a JEOL EX 1200 electron microscope operated at 80 kV.

For scanning electron microscopy, following dehydration, the samples were critical-point dried, mounted on copper stubs and sputter-coated with gold. Observations were made on a JEOL JSM 820 electron microscope operating at 20 kV.

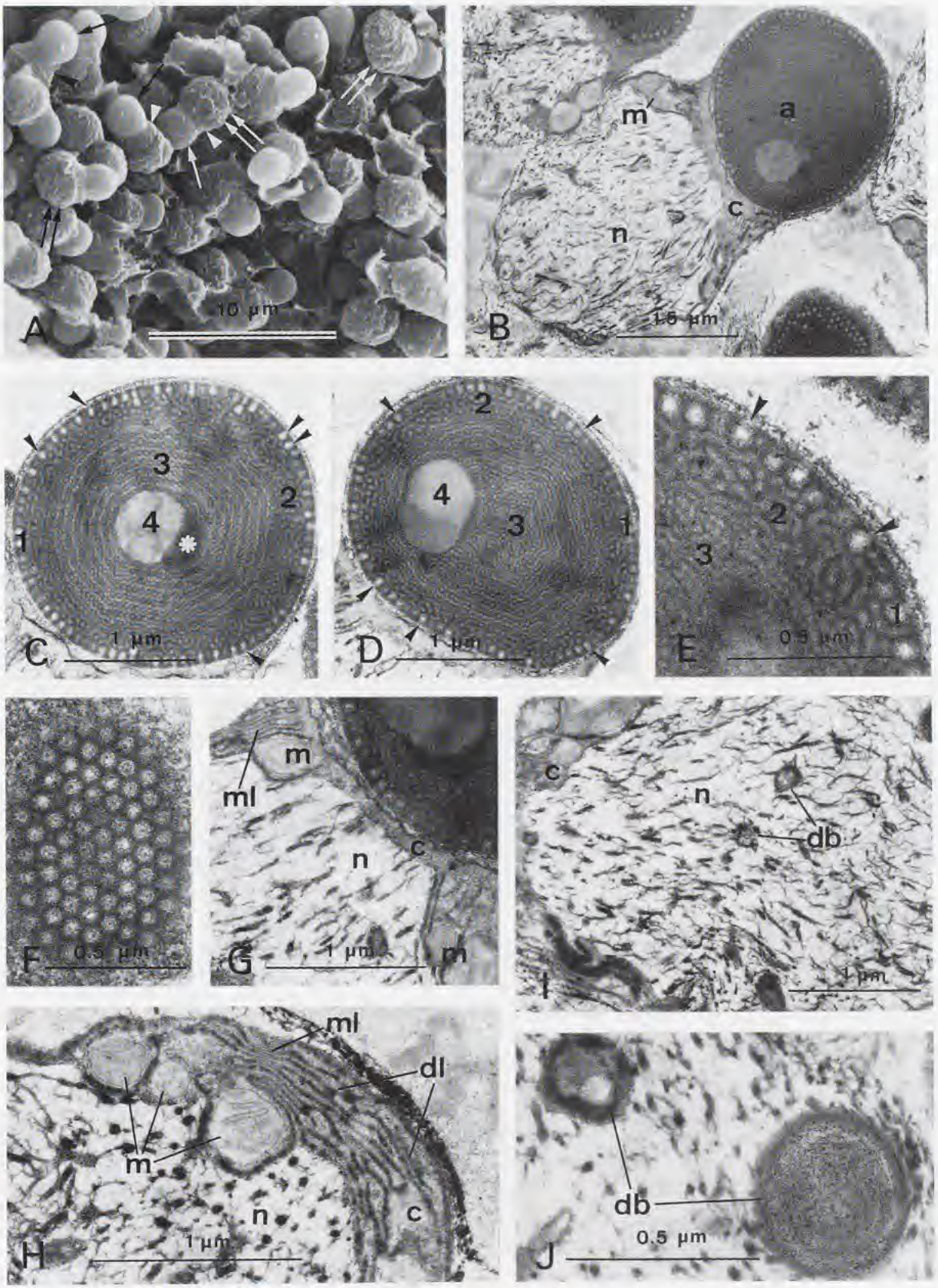
RESULTS

Spermatozoon of Aristeus antennatus

The spermatozoon of *Aristeus antennatus* basically comprises a conspicuous and prominent sperical acrosome and the nuclear structure (Figs 1a, b, 3a), the cytoplasmic mass being fairly reduced. The acrosome has a smooth surface, whereas the region containing the nucleus is more irregular in profile (Fig. 1a, b). The whole cell is 5.23 ± 0.33 µm (n = 16) in length. The acrosome measures 2.30 ± 0.16 µm (n = 19) in diameter and the nuclear region 3.02 ± 0.25 µm (n = 16) in width.

FIG. 1. — Scanning (**A**) and transmission (**B-J**) electron micrographs of *Aristeus antennatus* spermatozoa. **A:** Spermatozoa in the ampulla. The acrosome (arrows), cytoplasmic collar (arrowheads) and nuclear region (double arrows) are distinguished. **B:** Sagittal section of spermatozoon. **C-E:** Longitudinal sections of acrosomes showing the complex arrangement of their contents consisting of three distinct layers that correspond to the peripheral layer (1) of small clear vesicles (arrowheads), the marbled-like subperipheral zone (2), and the central region of concentrically arranged material (3). At the innermost region is a clear area (4) that usually appears associated with a dense osmiophilic substance (asterisk). **F:** Tangential section of acrosome through the peripheral vesicle layer. **G, H:** Sagittal (**G**) and oblique (**H**) sections of cytoplasmic collars containing mitochondria and membrane lamellae in continuity with osmiophilic dense lamellae. **I:** The nuclear region of a spermatozoon in sagittal section.. Dense bodies are embedded in the nucleoplasm. **J:** Detail of dense bodies showing myelin-like structure. a, acrosome; c, cytoplasmic collar; db, dense bodies; dl, dense lamellae; m, mitochondria; ml, membrane lamellae; n, nuclear region.

Source : MNHN, Paris



Source : MNHN, Paris

The acrosome is formed by a spherical acrosomal vesicle with structurally complex contents. No distinct subacrosomal region is found. The inner arrangement of the acrosome is symmetrical, since both longitudinal and cross sections show equivalent structural patterns. At the periphery is a single layer of electron-lucent vesicular structures which appear to originate from small invaginations of the acrosome membrane (Fig. 1c-e). Tangential sections show them to be regularly sized (about 80 nm in diameter) and densely packed (Fig. 1f). Immediately under the vesiculate layer, the acrosome materials take on a marbled appearance (Fig.1c, d). The central region of the acrosome is filled with a material arranged in concentric rows of apparently hollow spherical particles (Fig. 1c, d). In many sections, an electron-clear area is observed at the innermost region of the acrosome. It may be centrally located (Fig. 1c) or eccentric (Fig. 1d). A dense material is usually associated with this electron-clear zone (Fig. 1c).

The cytoplasm is reduced to a collar intervening between the acrosome and nucleus which extends laterally over the anterior third of the nuclear region (Figs 1a, b, 3a). The central portion of the cytoplasmic collar shows no noticeable substructure. In contrast, the lateral expansion contains parallel membrane arrays, osmiophilic lamellae and clusters of mitochondria (Fig. 1b, g, h). In favourable sections, osmiophilic lamellae are seen to be in continuity with membrane lamellae (Fig. 1h). Both elements are orientated in the direction of the lateral expansion of the cytoplasmic mass.

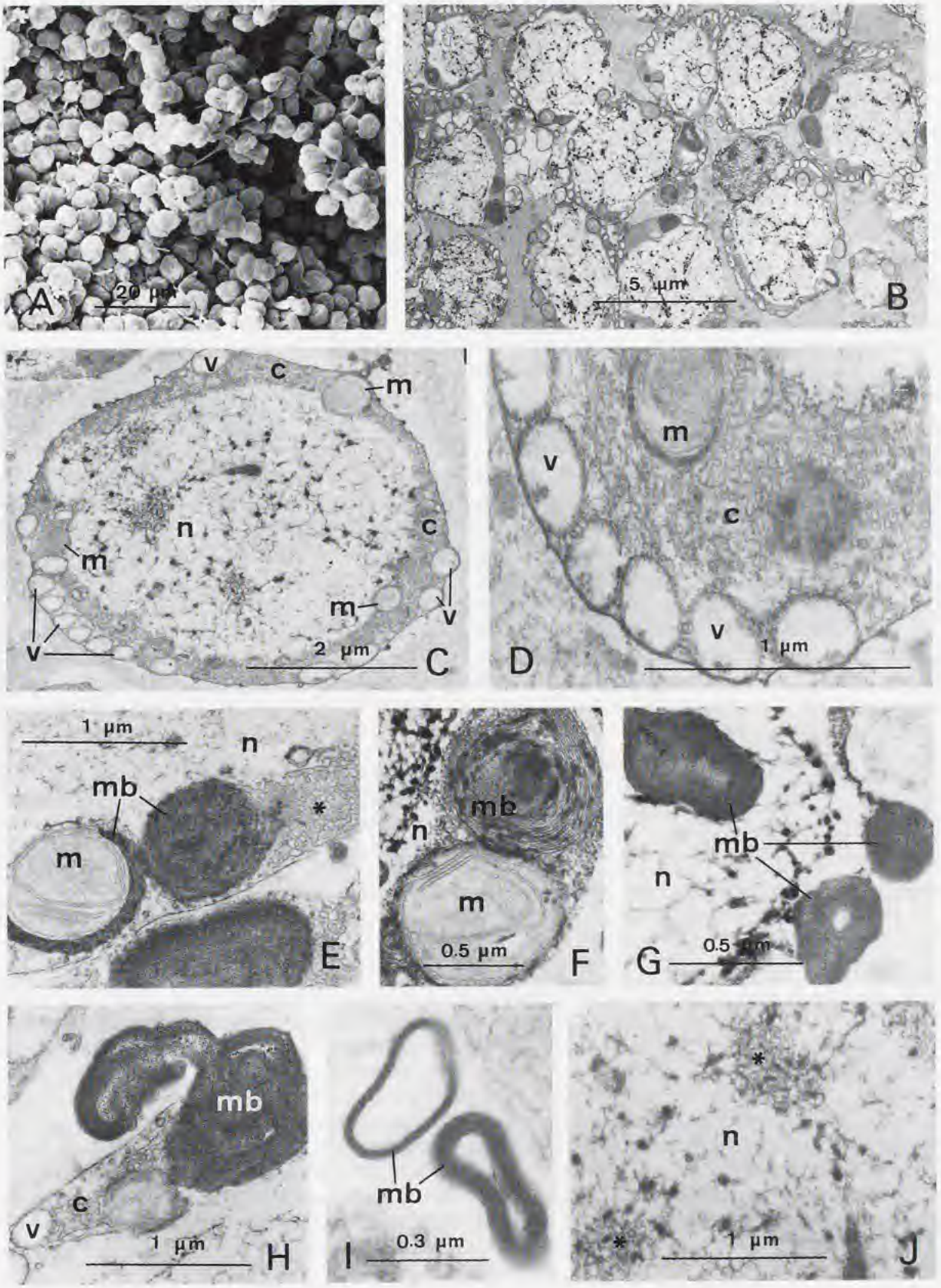
The nuclear region contains a non-membrane bound network of chromatin fibres (Figs 1b, h-j, 3a). Anteriorly, the chromatin is surrounded by the cytoplasm, and laterally and posteriorly solely by the plasma membrane. Many fibres appear inserted in the cytoplasmic mass, becoming orientated longitudinally (Fig. 1b, g). Dense bodies and occasional vesicles are observed in the nucleoplasm (Fig. 1i, j). The dense bodies are of membrane origin, since they sometimes show a myelin-like structure (Fig. 1j).

Spermatozoon of Aristaeomorpha foliacea

The sperm of *Aristaeomorpha foliacea* are roundish or slightly ellipsoid cells measuring 5.66 ± 0.79 µm (n = 33) at their widest point. Puzzingly, this dendrobranchiate sperm type does not exhibit any recognizable acrosomal structure (Figs 2a, b, 3b). Thus, the spermatozoon simply consists of the nuclear region and cytoplasm (Fig. 2c). The cytoplasm forms a peripheral band around the nucleoplasm and is bounded by the plasma membrane. The thickness of such a cytoplasmic band is irregular throughout the cell (Fig. 2b), sometimes having discontinuities in which the nucleoplasm is in direct contact with the plasma membrane. The main cytoplasmic elements are electron-lucent vesicles, mitochondria and myelin-like bodies (Fig. 2c-f). The cytoplasmic background mainly consists of granular material and anastomosing membranes (Fig. 2e). Numerous vesicles about 0.3 µm in size occur at the very periphery of the cytoplasmic band, often causing slight bumps in the sperm surface (Fig. 2c). As a result of this, the vesicle and plasma membranes are tightly apposed at the zone of contact of both (Fig. 2d). The peripheral clear vesicles contain a moderately electron-dense substance which either accumulates in a small core or forms a thin layer on the inner side of their membrane (Fig. 2d). Mitochondria occur in numbers of approximately 1-3 per section. They show randomly distributed cristae (Fig. 2 e, f).

FIG. 2. — Scanning (**A**) and transmission (**B-J**) electron micrographs of *Aristaeomorpha foliacea* spermatozoa. **A, B:** Sperm mass in the ampulla. **C:** Radial section of spermatozoon showing the peripheral cytoplasmic band and central nuclear region. **D-F:** Diverse details of peripheral cytoplasm to show the main cytoplasmic elements: peripheral electron-clear vesicles, mitochondria, dense myelin-like bodies and anastomosing membranes (asterisk). **G-I:** Myelin-like bodies appear to be released from the cytoplasm and occasionally may occur either out of the cell (**I**) or in the nucleoplasm (**G**). **J:** The nucleoplasm frequently shows nodules (asterisks) where the chromatin fibres become densely entangled. c; perinuclear cytoplasm; m, mitochondria; mb, myelin-like bodies; n, nuclear region; v, peripheral vesicles.

Source : MNHN, Paris



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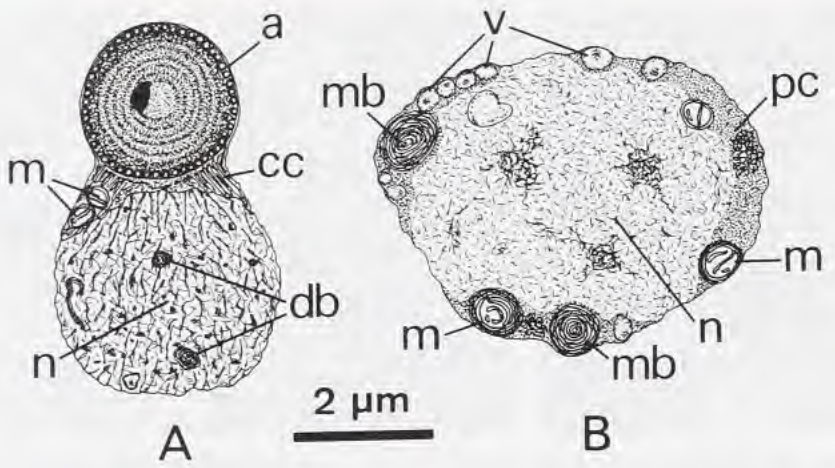

FIG. 3. — **A:** Spermatozoon of *Aristeus antennatus*. **B:** Spermatozoon of *Aristaeomorpha foliacea*. Drawn from transmission electron micrographs. a, acrosome; cc, cytoplasmic collar; db, dense bodies; m, mitochondria; mb, myelin-like bodies; n, nuclear region; pc, perinuclear cytoplasm; v, peripheral vesicles.

Myelin-like bodies apparently originate by coalescence of waste cytoplasmic membranes into concentric layers. Frequently, membrane layers become deposited around (Fig. 2e) or constitute dense bodies in close association with mitochondria (Fig. 2e, f). Isolated myelin-like bodies can be encountered not only in the cytoplasmic band, but also within the nucleoplasm (Fig. 2g). Some images suggest that at least some of them are extruded at the cell periphery (Fig. 2g-i).

As typical for all dendrobranchiates, the chromatin of *A. foliacea* sperm shows an uncondensed reticulate pattern and is not bounded by a nuclear envelope. It is characteristic that in this species nodules where chromatin fibres become more densely entangled are present (Fig. 2j).

DISCUSSION

The sperm morphologies of the Aristeidae *Aristeus antennatus* and *Aristaeomorpha foliacea* are markedly inconsistent with the dendrobranchiate unistellate sperm plan. While the unistellate spermatozoa of most dendrobranchiates consist of a main body housing the nucleus and an acrosomal complex formed by cap region and prolonging spike (see reviews by FELGENHAUER & ABELE [5], JAMIESON [9], KROL *et al.* [11]), the sperm of *A. antennatus* have a spike-less acrosome and those of *A. foliacea* lack any acrosomal structure at all.

Unlike the other studied dendrobranchiates, the spermatozoa of *Aristeus antennatus* lack a perinuclear cytoplasmic band, so that the nucleoplasm is bounded by only the plasma membrane in most of the nuclear profile. In gross morphology, these features liken the *A. antennatus* spermatozoon to that of reptant Pleocyemata, in particular Anomura [7, 8, 18, 25, 26] and Astacidea [2, 19, 24], in which the acrosome is prominent and lies fairly apart from the posterior nuclear region, with the cytoplasmic region located in between. Albeit these features are common to *A. antennatus* sperm, the spherical acrosome of the latter has no basal invagination as does the acrosomal vesicles in the above-noted reptants. In such a respect, this spermatozoon would be similar to that of the Palinuridae [13, 23]. Nevertheless, in spite of the several coincidences, there exist notable dissimilarities with regard to the general reptant sperm morphology, such as the absences of nuclear envelope and nuclear arms or the different substructure of the acrosome.

From the above comments, it is concluded that the spermatozoon of *Aristeus antennatus* simultaneously blends typically dendrobranchiate and typically reptantian characteristics, whereby its placement into one of the two traditionally established decapod sperm categories becomes

Source : MNHN, Paris

difficult. Available ultrastructural descriptions of spermatids in this species [4] reveal a mechanism of acrosome formation resembling the one observed in many species of Pleocyemata [1, 6, 10, 12, 13, 15-18, 20]. However, it slightly differs from that described for the only two other Dendrobranchiata in which spermiogenesis has been ultrastructurally investigated, *Sicyonia ingentis* (see [21]) and *Parapeneus longirostris* (see [14]). Actually, the complex ultrastructural arrangement of the acrosomal vesicle contents in *A. antennatus* mature sperm is not comparable with that of other decapods. The absence of either a spike or any other significant structure at the place where the spike should supposedly lie, as well as the spherical (not capping) acosome with varied contents, are to be considered as clear autapomorphies within the Dendrobranchiata. Another assumed apomorphic character is the subacrosomal (instead of the plesiomorphic perinuclear) cytoplasm.

The most significant feature of the ellipsoid, appendage-less sperm of *Aristaeomorpha foliacea* is the amazing absence of any kind of acrosomal structure, which is unique in the Dendrobranchiata. Despite thorough examination of spermatozoa from both vasa deferentia from males and seminal receptacles from females, no trace of any structure that may be related to an acrosome was found. Hitherto, acrosome lack in Eumalacostraca was only known in Euphausiacea [9] and in Decapoda Stenopodidea [5]. As up to date *A. foliacea* is the only known case of dendrobranchiate sperm lacking the acrosome, one would be inclined to infer that this pattern has been reached by simplification from a peneoid-like (spiked) spermatozoon through evolutionary loss of the acrosome. Nevertheless, the findings of acrosome-less spermatozoa resembling the one of *A. foliacea* in Euphausiacea [9], Stenopodidea [5] and Sergestoidea [see MEDINA, this volume] suggest that the absence of the acrosome would be a primitive condition in Eucarida. Therefore, if the absence of the acrosome is plesiomorphic, the acrosome of *Aristeus antennatus* sperm represents a secondary acquisition, hence an apomorphy, within the Aristeidae. On the other hand, the spiked acrosome would be a synapomorphy of Penaeidae and Sicyonidae.

Well-known spermatozoal symplesiomorphic features of the Decapoda are the diffuse chromatin, partial or total disruption of the nuclear envelope and absence of flagellum. In addition, the present study confirms as clear plesiomorphies in Dendrobranchiata: a) non-membrane bound nuclear region, b) filamentous chromatin, c) perinuclear cytoplasm, d) absence of centrioles and radial arms. The reduced subacrosomal distribution of the cytoplasm in *Aristeus antennatus* should thus be considered as an autapomorphy. In conclusion, two distinct evolutionary trends appear to have occurred in Aristeidae, leading to sperm morphologies which are distant from the dendrobranchiate sperm plan exemplified by the assemblage Penaeidae-Sicyonidae. This suggests that, although ultrastructural studies on crustacean spermatozoa are generally useful for phylogenetic approaches, one must be cautious in certain instances, since notable variation may occur in close taxa [5].

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Source : MNHN, Paris

Ultrastructure and Phylogeny of the Spermatozoa of the Infraorders Thalassinidea and Anomura (Decapoda, Crustacea)

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ABSTRACT

The spermatozoal morphology of 62 species of anomuran and thalassinidean decapod crab (including many species for which the spermatozoal ultrastructure is previously undescribed) is compared at the light and electron microscope level. Relationships between taxa are postulated on the basis of shared spermatozoal characters and comparisons made with existing ideas about the relationships and evolution of these taxa. Pronounced spermatozoal differences between representatives of some families in the Infraorder Thalassinidea support the separation and reclassification of this group into three superfamilies. In contrast, within the anomuran superfamily Paguroidea, the families Paguridae, Parapaguridae, Diogenidae and Coenobitidae are shown to be united by a suite of ultrastructural spermatozoal characters. Each of these families, however, can be distinguished by characteristic spermatozoal features. The anatomically diverse family Diogenidae exhibits a range of sperm morphologies intermediate between the Paguridae and Coenobitidae which may indicate a diphyletic origin for this family. The families Galatheidae and Porcellanidae, within the superfamily Galatheoidea, each show a set of distinctive spermatozoal characters, but share very few characters.

RÉSUMÉ

Ultrastructure et phylogénie des spermatozoïdes des sous-ordres Thalassinidea et Anomura (Decapoda, Crustacea).

La morphologie du spermatozoïde de 62 espèces de Crustacés Décapodes Anomoures et Thalassinides, (y compris de nombreuses espèces dont le spermatozoïde n'avait jamais été décrit), est comparée en microscopie photonique et électronique. Les relations entre les taxons sont postulées sur la base de caractères spermatologiques partagés et des comparaisons sont faites avec les idées pré-existantes concernant leurs relations phylétiques et leur évolution. Les différences importantes entre les spermatozoïdes des représentants de quelques familles du sous-ordre Thalassinidea soutiennent la séparation et la re-classification de ce groupe en trois super-familles. Au contraire, à l'intérieur de la super-famille des Paguroidea, on montre que les familles Paguridae, Parapaguridae, Diogenidae et Coenobitidae sont unies par un ensemble de caractères ultrastructuraux du spermatozoïde. Toutefois, chacune de ces familles peut être distinguée par des caractéristiques spermatologiques. La famille Diogenidae, qui est anatomiquement diversifiée, montre des morphologies spermatiques intermédiaires entre les Paguridae et les Coenobitidae, qui pourrait indiquer le diphylétisme de son origine. Les familles Galatheidae et Porcellanidae, membres de la super-famille Galatheoidea, montrent chacune un ensemble de caractères spermatologiques distinctifs, mais ont peu de caractères en commun.

TUDGE, C. C., 1995. — Ultrastructure and phylogeny of the spermatozoa of the infraorders Thalassinidea and Anomura (Decapoda, Crustacea). *In*: JAMIESON, B. G. M., AUSIO, J., & JUSTINE, J.-L. (eds), Advances in Spermatozoal Phylogeny and Taxonomy. *Mém. Mus. natn. Hist. nat.*, **166** : 251-263. Paris ISBN : 2-85653-225-X.

Source : MNHN, Paris

Within the order Decapoda, the infraorder Anomura has undergone considerable revision since its introduction [27]. Classifications of the Anomura such as those of GLAESSNER [17] and BORRADAILE [3], recognize the four superfamilies, Thalassinoidea, Paguroidea, Galatheoidea and Hippoidea. More recently the thalassinoids have been excluded from the Anomura and the constituent anomuran superfamilies redefined as the Paguroidea, Lomoidea, Galatheoidea and Hippoidea [30, 31].

Some significant taxonomic changes have been proposed for the infraorder Thalassinidea and the group now comprises three superfamilies and is considered a monophyletic taxon, distinct from the Anomura [40]. In the past the thalassinids have been linked with the anomurans on the basis of larval morphology [18, 26], and adult somatic characters [28]. The thalassinids are considered to be an important pivotal group in the evolution and phylogeny of the other decapod infraorders [2, 6].

The superfamily Paguroidea consists of the families, Coenobitidae, Diogenidae, Paguridae, Parapaguridae, Pylochelidae and Lithodidae [30]. All except the Lithodidae are considered true hermit crabs [5] but recently it has been shown that the lithodids may have close links with the genus *Pagurus* in the Paguridae [13].

The superfamily Lomoidea contains the monospecific genus *Lomis* in the family Lomidae. Once considered a symmetrical hermit crab [4], this enigmatic crab has since been elevated to its own family and superfamily [29, 34] but continues to be problematic in regard to its relationship to the remainder of the Anomura [28].

The superfamily Galatheoidea contains the families Aeglidae, Chirostylidae, Galatheidae and Porcellanidae, of which the Aeglidae, containing the single genus *Aegla*, are ecologically (restricted to freshwater) and morphologically distinct [28].

The superfamily Hippoidea (the mole crabs) contains only two families, Albuneidae and Hippidae, which have been variously allied with the other families of the Anomura but appear to be very distinct. They have been postulated to be more closely related to the thalassinoids than to the Anomura [28].

The species of thalassinidean and anomuran crabs studied for spermatozoal morphology (light or transmission electron microscopy) and published up to 1994 are listed in Table 1. This list comprises 32 species from 17 genera in 9 families. A further 30 species have been investigated for spermatozoal morphology by the author (Table 2) and this brings the total number of thalassinidean and anomuran taxa for which the spermatozoal structure is known to 62 species from 33 genera in 15 families. In addition to the listed publications, the ultrastructure of anomuran spermatozoa is briefly covered in several general crustacean sperm reviews [16, 23, 25].

MATERIALS AND METHODS

The crab specimens were collected from a wide range of localities including Australia, the South-West Pacific, the Mediterranean and European waters. The testes and ducts of the vasa deferentia were removed from the crabs and fixed in cold (4°C) glutaraldehyde for a minimum of 2 hours, after which the remainder of the standard techniques for transmission electron microscopy [44] were carried out. This procedure was undertaken for all species except for *Thalassina squamifera*, which was deep frozen, thawed and fixed in neutral buffered formalin before removal of the testes and then subjected to the standard TEM fixation procedure. Micrographs were taken on Hitachi H-300, JEOL 100-s and Hitachi H-600 transmission electron microscopes at 80, 60 and 75 kV respectively.

RESULTS

Infraorder Thalassinidea

The spermatozoa of the three investigated taxa in this group are each different in morphology, although the sperm cells of *Callianassa australiensis* and *Axius glyptocercus* are more similar to each other than to those of *Thalassina squamifera*. The sperm cells of

Source : MNHN, Paris

TABLE 1. — Thalassinidean and anomuran taxa previously investigated for spermatozoal morphology.

Infraorder Thalassinidea	
Superfamily Callianassoidea	
Family Upogebiidae	
Upogebia pusilla	[24] (as *Gebia littoralis*)
Superfamily Axioidea	
Family Calocarididae	
Calocaris macandreae	[41]
Infraorder Anomura	
Superfamily Paguroidea	
Family Coenobitidae	
Birgus latro	[23; 44; 45]
Coenobita clypeatus	[20; 21; 44]
Coenobita rugosus	[32]
Coenobita variabilis	[44] (as *C. spinosus*)
Family Diogenidae	
Clibanarius corallinus	[23; 44]
Clibanarius erythropus	[24] (as *C. misanthropus*); [46]
Clibanarius longitarsus	[14; 15; 32]
Clibanarius nathi	[32]
Clibanarius taeniatus	[44]
Clibanarius virescens	[44]
Dardanus arrosor	[24] (as *Pagurus striatus*)
Dardanus crassimanus	[44]
Dardanus megistos	[32] (as *Pagurus punctulatus*)
Diogenes custos	[44]
Diogenes miles	[15]
Paguristes oculatus	[24] (as *P. maculatus*)
Family Lithodidae	
Lithodes maja	[41]
Family Paguridae	
Pagurus bernhardus	[7-12; 35-39; 41] (as *Eupagurus bernhardus*)
Pagurus excavatus	[24] (as *Eupagurus angulatus*)
Pagurus prideaux	[24] (as *Eupagurus prideauxii*)
Superfamily Galatheoidea	
Family Galatheidae	
Allogalathea elegans	[23] (as *Allogalathea* sp.)
Galathea squamifera	[24; 41]
Galathea strigosa	[24]
Munida rugosa	[24]
Pleuroncodes planipes	[22]
Family Porcellanidae	
Petrolisthes lamarckii	[23]
Pisidia longicornis	[41] (as *Porcellana longicornis*)
Superfamily Hippoidea	
Family Hippidae	
Emerita analoga	[48-52]
Emerita asiatica	[43]
Emerita talpoida	[1; 33]

Source : MNHN, Paris

TABLE 2. — New thalassinidean and anomuran species investigated by the author for spermatozoal morphology.

Infraorder Thalassinidea

Superfamily Thalassinoidea
Family Thalassinidae
Thalassina squamifera

Superfamily Callianassoidea
Family Callianassidae
Callianassa australiensis
C. arenosa

Superfamily Axioidea
Family Axiidae
Axius glyptocercus

Infraorder Anomura

Superfamily Paguroidea
Family Coenobitidae
Coenobita brevimanus
C. perlatus
C. purpureus
Family Diogenidae
Calcinus gaimardii
C. laevimanus
C. minutus
Cancellus sp.
Dardanus lagopodes
D. scutellatus
Dardanus sp.
Diogenes pallescens
G. n. sp. n.(cf. *Trizopagurus strigimanus*)
Family Paguridae
Pagurus chevreuxi
P. hirtimanus
Porcellanopagurus sp.
Xylopagurus sp.
Family Parapaguridae
Sympagurus sp.

Superfamily Lomoidea
Family Lomidae
Lomis hirta

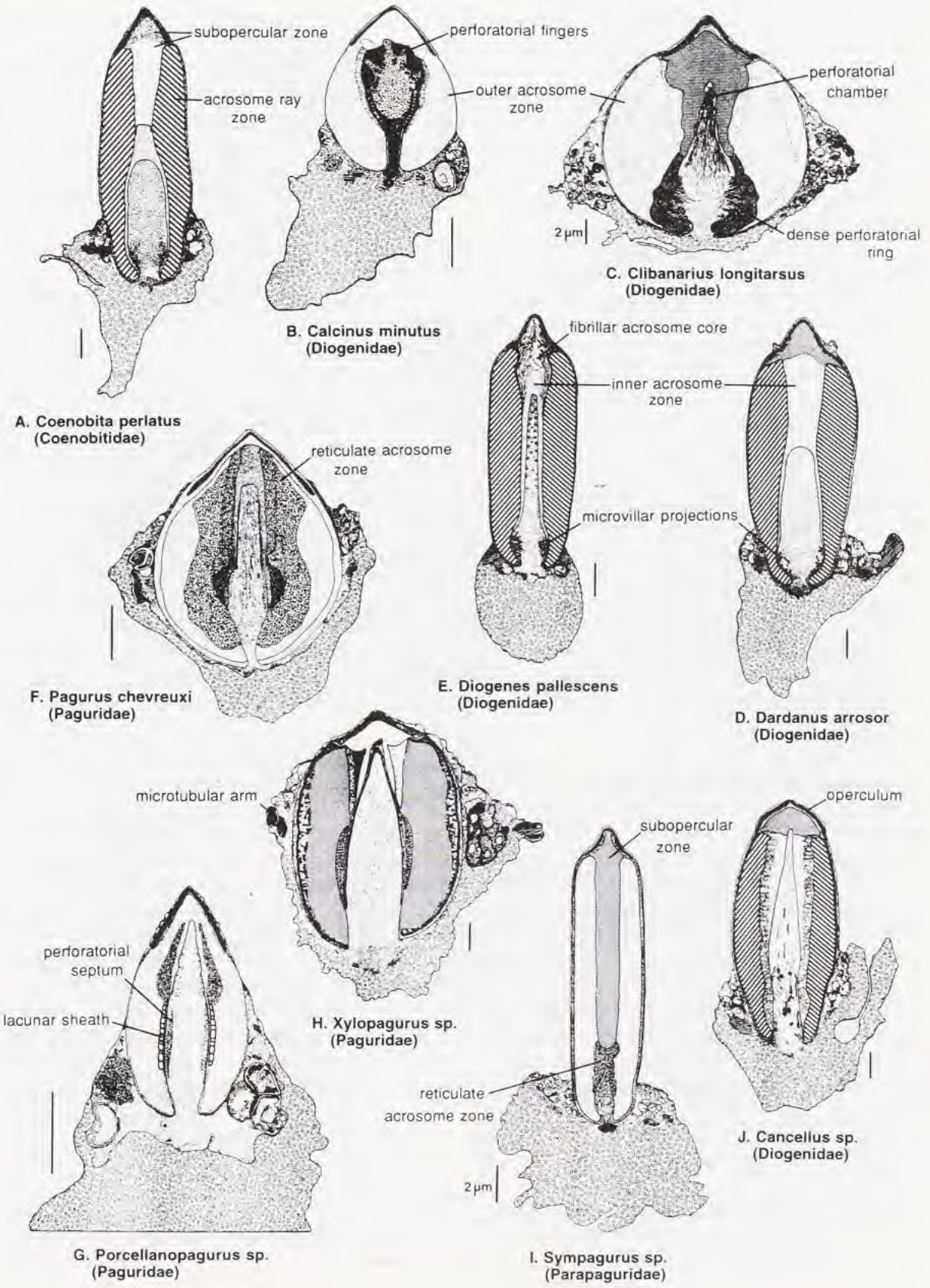
Superfamily Galatheoidea
Family Chirostylidae
Eumunida sternomaculata
Uroptychus sp.
Family Galatheidae
Munida sp.
Munidopsis sp.
Family Porcellanidae
Aliaporcellana suluensis
Petrolisthes armatus
Polyonyx transversus

Superfamily Hippoidea
Family Hippidae
Hippa pacifica

C. australiensis and *A. glyptocercus* are spherical with prominent microtubular spines (probably four) radiating from the equatorial region. The sperm cell can be divided into two hemispheres, with the upper containing the cytoplasmic organelles and an electron-dense acrosome vesicle with operculum, while the lower hemisphere is composed of the nucleus (Fig. 2a). The microtubular spines originate in the nucleus and appear to pass completely through it. *A. glyptocercus* differs from *C. australiensis* in having a large columnar invagination (perforatorial chamber) penetrating the sperm cell from the posterior or lower pole to a subterminal position below the operculum (Fig. 2a). The spermatozoa of *T. squamifera* differs markedly in being composed of a large, ovoid, concentrically zoned acrosome vesicle, capped by a tri-layered operculum and posteriorly embedded in a ring of cytoplasm and nucleus (Fig. 2b). A number of microtubular arms originate in the posterior nucleus. The acrosome vesicle is penetrated posteriorly by a columnar perforatorial chamber which terminates below the operculum and appears to have an electron-dense thickened ring around its base.

FIG. 1. — **A-J:** Semidiagrammatic representations of longitudinal sections of hermit crab spermatozoa. Homologous regions between sperm cells are similarly shaded. Drawings based on tracings of micrographs. Scale bars = 1 µm (except where indicated).

Source : MNHN, Paris



Source : MNHN, Paris

Infraorder Anomura

Family Coenobitidae. The spermatozoa of *Birgus latro* and the six investigated species in the genus *Coenobita* are all very similar in morphology [23, 44, 45]. The concentrically zoned acrosome vesicle is a large and oblong-ovoid (*Birgus*) to cylindrical (*Coenobita*) structure, penetrated posteriorly by a columnar perforatorial chamber in which the walls are drawn out into microvillar projections (Fig. 3a). The anterior pole of the acrosome vesicle is capped by a domed operculum, below which a divided subopercular zone occurs (Fig. 1a). The acrosome is largely composed of an acrosome ray zone which has the appearance of radiating dark and light bands (Fig. 3b). The acrosome vesicle is cupped posteriorly by the cytoplasm (with three microtubular arms) and nucleus.

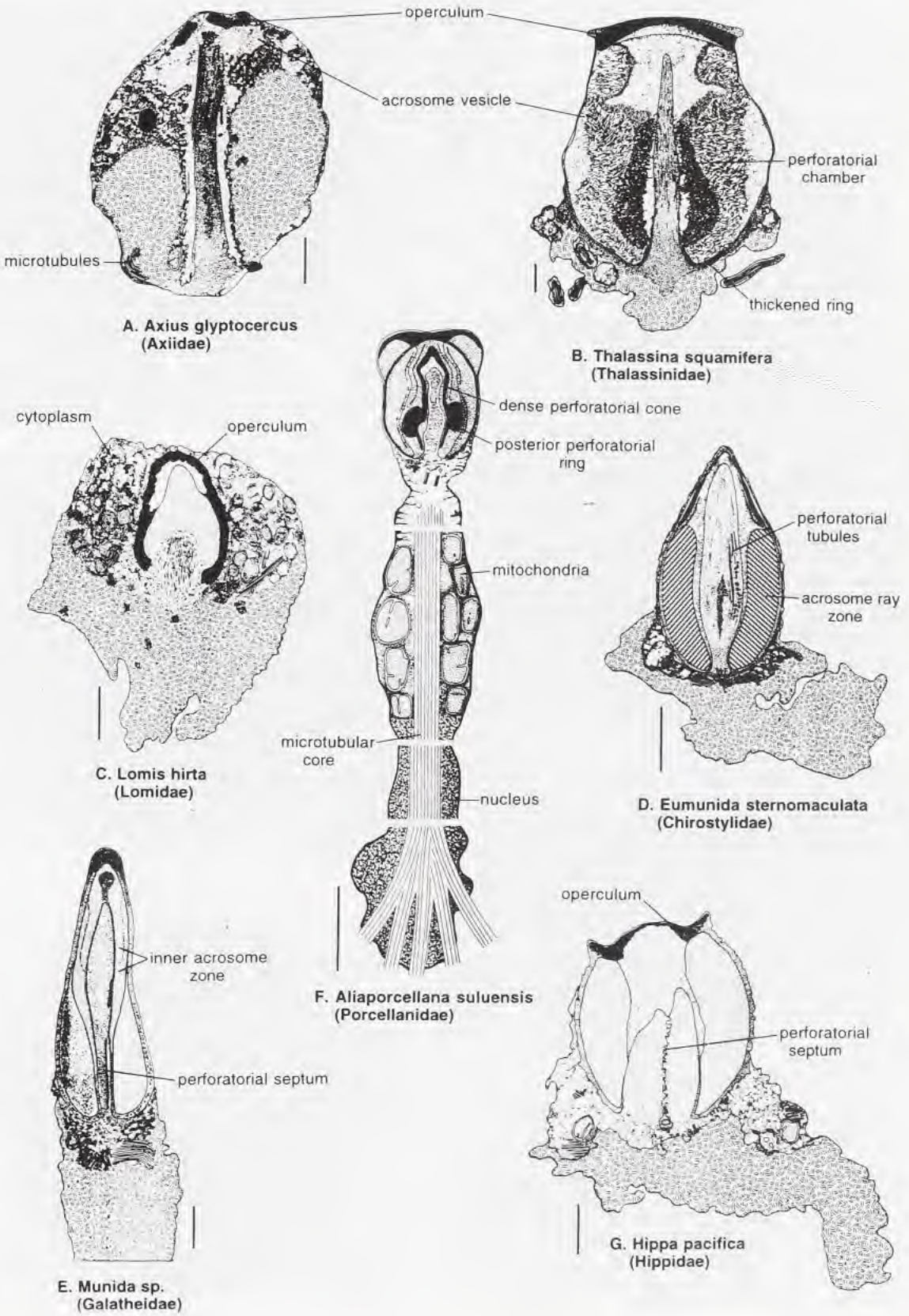
Family Diogenidae. The spermatozoa in the genus *Calcinus* are composed of a spherical acrosome vesicle, capped by a domed operculum, and posteriorly penetrated by a perforatorial chamber (Fig. 1b). The acrosome is embedded in the cytoplasm and nucleus and the perforatorial chamber walls project laterally to form short microvillar projections. An autapomorphy for this genus is the splitting of the anterior end of the perforatorial chamber into two or more fingers (Fig. 3c). The genus *Cancellus* has a spermatozoon with a cylindrical acrosome vesicle with a conspicuous acrosome ray zone, which is penetrated by a large tapering perforatorial chamber (Fig. 1j). Although perforatorial tubules are present in the chamber, there do not appear to be any microvillar projections and the operculum is unique among the paguroids in having a central perforation (Fig. 3d). The spermatozoa of the genus *Clibanarius* have an ovoid acrosome vesicle penetrated by a perforatorial chamber with microvillar projections but the chamber is distinctive in having a bulbous posterior region and a thin anterior projection. A dense ring (autapomorphy) occurs around the bulbous region of the perforatorial chamber (Fig. 1c). The sperm cells of the genus *Dardanus* are similar to the spermatozoa of the coenobitids but the acrosome vesicle (with conspicuous acrosome ray zone) (Fig. 3b) is generally shorter and more ovoid in shape and the subopercular zone is not divided into two distinct regions (Fig. 1d). The spermatozoa of the genus *Diogenes* are characterised by modification of the inner acrosome zone into a fibrillar core structure. The perforatorial chamber possesses microvillar projections and has the posterior bulb and anterior projection, like in clibanarids, but there is a prominent acrosome ray zone as in the coenobitids and dardanids (Fig. 1e).

Family Paguridae. The spermatozoa of the genus *Pagurus* lack microvillar projections in the perforatorial chamber, generally have ovoid acrosome vesicles with one or more reticulated acrosome zones (Fig. 3e) and the perforatorial chamber has a bulbous posterior region and a tapered anterior projection (Fig. 1f). *Porcellanopagurus* spermatozoa have a similarly shaped acrosome vesicle and perforatorial chamber (without microvillar projections) to *Pagurus* but the bulbous region of the perforatorial chamber has the walls sculpted into longitudinal septa and is surrounded by a vesiculated (lacunar) sheath (Fig. 1g). The spermatozoa of *Xylopagurus* differ from the other pagurids in having the microtubular arms emerging from the cytoplasm in the anterior part of the sperm cell and not posteriorly (this also differs from all other paguroids) and in possessing a larger, more cylindrical acrosome vesicle with unusual zonation (Fig. 1h).

Family Parapaguridae. The spermatozoa of *Sympagurus* share the bulbous shape of the posterior region of the perforatorial chamber, the lack of microvillar projections and the presence of a reticulated acrosome zone with members of the family Paguridae but differ in having the acrosome vesicle extended anteriorly to more than three times the length of any pagurid (Fig. 1i).

FIG. 2. — **A-H:** Semidiagrammatic representations of longitudinal sections of thalassinidean and anomuran crab spermatozoa. Homologous regions between sperm cells are similarly shaded. Drawings based on tracings of micrographs. Scale bars = 1 μm.

Source : MNHN, Paris



Source : MNHN, Paris

Family Lomidae. The only representative in this family, *Lomis hirta*, has a spermatozoal morphology which is different from that of all other anomurans. The sperm cell is irregular, but basically globular, with one to three vertices which may be extended into microtubular arms (two arms have been observed in a single sperm but often three vertices are apparent). The acrosome vesicle is an inverted cup shape, penetrated by a perforatorial chamber, and is completely embedded in the cytoplasm. A discontinuous, electron-dense zone (interpreted as the operculum) extends around the entire acrosome vesicle (Fig. 2c).

Family Chirostylidae. The investigated genera in this family, *Eumunida* and *Uroptychus*, have spermatozoa with a spherical to ovoid acrosome vesicle, capped by a domed operculum and penetrated by a perforatorial chamber. The walls of the perforatorial chamber lack microvillar projections, but some perforatorial tubules are present in *Eumunida* (Fig. 2d). An acrosome ray zone is present in the posterior region of the acrosome vesicle in both.

Family Galatheidae. The spermatozoal morphology of *Allogalathea*, *Munida* and *Munidopsis* differs slightly in each species, but there are some characteristic features of the family. The acrosome vesicles are elongate and generally cylindrical, are capped by a domed operculum and penetrated for most of their length by a perforatorial chamber. The inner acrosome zone is divided into two regions of differing electron-density, the outer acrosome zone is situated posteriorly in the acrosome vesicle, and longitudinally arranged perforatorial septa are present in the posterior region of the perforatorial chamber (Fig. 2e).

Family Porcellanidae. The spermatozoal morphology found in the investigated members of this family can be divided into two types. In *Petrolisthes* the nucleus is globular in shape and the cytoplasm forms a thin, neck-like region between it and the acrosome while in the remaining genera, *Aliaporcellana*, *Pisidia* and *Polyonyx*, the sperm cells are elongate, with the cytoplasm and nucleus forming a veneer over a central microtubular core (Fig. 2f). The microtubular core splits posteriorly to form several external microtubular arms (Fig. 3f). The sperm cells of each have a complexly arranged, concentrically zoned acrosome vesicle, capped by a perforate operculum, penetrated by a perforatorial chamber (in which the walls are folded into septa) and share acrosomal features such as a dense perforatorial cone and posterior ring (Fig. 2f).

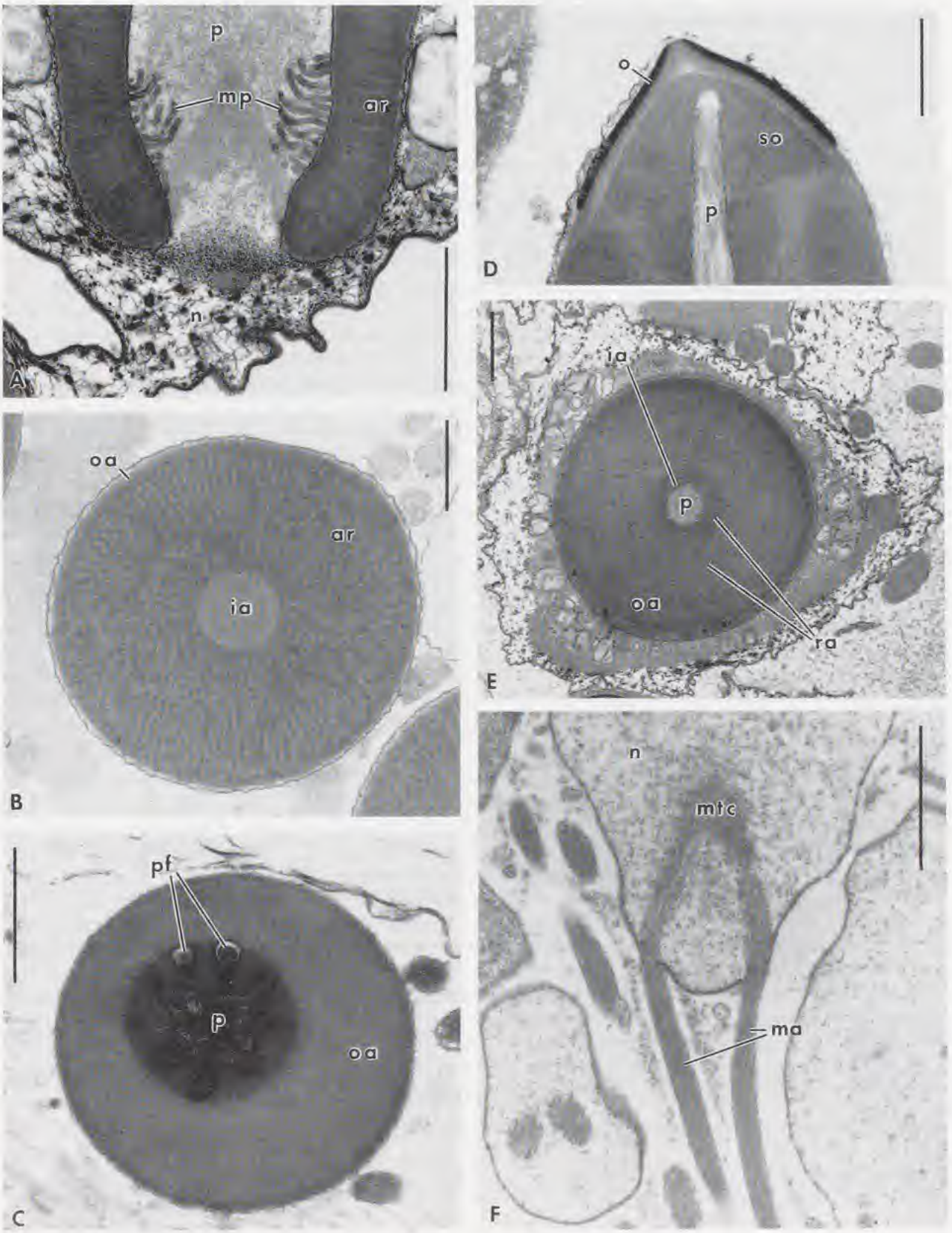
Family Hippidae. The sperm cell of *Hippa pacifica* is composed of a spherical to ovoid acrosome vesicle, capped by a broad operculum and penetrated by a wide perforatorial chamber. The unusual features of this spermatozoon are that the anterior end of the perforatorial chamber forms several tapered points, a membranous septum longitudinally divides the chamber and there are possibly up to five microtubular arms (Fig. 2g).

DISCUSSION

A recent reclassification of the infraorder Thalassinidea [40] has divided this group into three superfamilies. The available data from spermatozoal morphology would seem to vindicate this new arrangement, with *Thalassina*, *Callianassa* and *Axius* (from each of the superfamilies)

FIG. 3. — **A-F:** Transmission electron micrographs of longitudinal sections (LS) and transverse sections (TS) of spermatozoa of selected anomuran crabs. **A:** *Coenobita perlatus*. LS of perforatorial chamber showing microvillar projections. **B:** *Dardanus arrosor*. TS of acrosome vesicle showing conspicuous acrosome ray zone. **C:** *Calcinus minutus*. TS through upper acrosome vesicle showing splitting of the perforatorial chamber into separate fingers. **D:** *Cancellus* sp. LS of opercular region. **E:** *Pagurus chevreuxi*. TS through the acrosome vesicle showing reticulated acrosome zones. **F:** *Polyonyx transversus*. Oblique section through the posterior end of the nucleus showing the splitting of the microtubular core into discrete microtubular arms. Abbreviations: ar, acrosome ray zone; ia, inner acrosome zone; ma, microtubular arm; mp, microvillar projection; mtc, microtubular core; n, nucleus; o, operculum; oa, outer acrosome zone; p, perforatorial chamber; pf, perforatorial fingers; ra, reticulated acrosome zone; so, subopercular zone. Scale bars = 1 μm.

Source : MNHN, Paris



Source : MNHN, Paris

showing a different spermatozoal ultrastructure. The overall sperm morphology for the Thalassinidea is sufficiently different from that of the Anomura to support the separation of the two based on evidence from somatic morphology [28]. Of interest is the fact that the spermatozoa of *Thalassina* possess an apparent thickened ring (Fig. 2b) which, so far, has been recorded only in heterotreme and thoracotreme brachyurans [23].

Within the superfamily Paguroidea, the investigated members of the Coenobitidae and Diogenidae share many spermatozoal characters, but many genera possess apomorphies which distinguish them. In the Diogenidae, the genus *Calcinus* has the splitting of the perforatorial chamber into separate fingers (Figs 1b, 3c), *Cancellus* sp. has a perforate operculum (Figs 1j, 3d), the clibanarids possess the dense perforatorial ring (Fig. 1c), and *Diogenes* species have modified the inner acrosome zone into a fibrillar core structure (Fig. 1e). Members of the genus *Pagurus* (Figs 1f, 3e), in the Paguridae, share the shape of the perforatorial chamber, the absence of microvillar projections and the reticulated acrosome zone with *Sympagurus* (Fig. 1i) in the Parapaguridae, but the other genera (*Porcellanopagurus* and *Xylopagurus*) appear more distinctive (Fig. 1g, h). The light microscope observation of *Lithodes* (Lithodidae) [41] is the only spermatozoal information available on this family. It shows a sperm cell with a spherical, concentrically zoned acrosome vesicle, penetrated by a perforatorial chamber, and three conspicuous microtubular arms. This description places it within the Paguroidea but an ultrastructural study is necessary to investigate the claim that the lithodids have close links with the genus *Pagurus* in the Paguridae [13].

The superfamily Lomoidea contains the monospecific genus *Lomis* in the family Lomidae. The spermatozoon of *Lomis* possesses spermatozoal characters, such as microtubular arms (possible three?) and an acrosome vesicle penetrated by a perforatorial chamber (Fig. 2c), which justify its position in the Anomura but its sperm morphology is distinct enough to confirm placement in its own family and superfamily [29].

Each of the three investigated families, Chirostylidae, Galatheidae and Porcellanidae, in the superfamily Galatheoidea shows a particular spermatozoal morphology which appears characteristic for that family. The chirostylid spermatozoal morphology is more similar to that of hermit crabs than to any other galatheoid, particularly in the shape of the acrosome vesicle and the possession of an acrosome ray zone (Fig. 2d). The more elongate (fusiform) acrosome vesicle shape, division of the inner acrosome zone and the presence of septa in the perforatorial chamber characterise the members of the Galatheidae (Fig. 2e). The investigated members of the Porcellanidae all show a particular suite of acrosome vesicle characters which unite them but the overall sperm cell morphology divides the group. The globular nuclear form in *Petrolisthes* species clearly differentiates this genus from the other investigated genera which have an elongate sperm cell (Fig. 2f), reminiscent of flagellate spermatozoa. This basic division of the Porcellanidae is supported by larval [42, 47] and adult somatic morphology [19].

In the superfamily Hippoidea only representatives from the family Hippidae have been investigated for spermatozoal morphology. The spermatozoa observed in the genus *Emerita* (Table 1) have a more elongate acrosome vesicle than that described for *Hippa*. Though showing spermatozoal characteristics which place them in the Anomura (microtubular arms and acrosome vesicle structure), it is difficult to ally hippids with any other anomuran superfamily. The broad, horizontal operculum (Fig. 2g) has its nearest counterpart in members of the Porcellanidae (Fig. 2f) (though perforate in the latter case), or perhaps that seen in the thalassinidean, *Thalassina* (Fig. 2b). This last comparison may provide some evidence to support the statement that the hippids are the nearest relatives to the thalassinoids [28].

Source : MNHN, Paris

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Source : MNHN, Paris

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Source : MNHN, Paris

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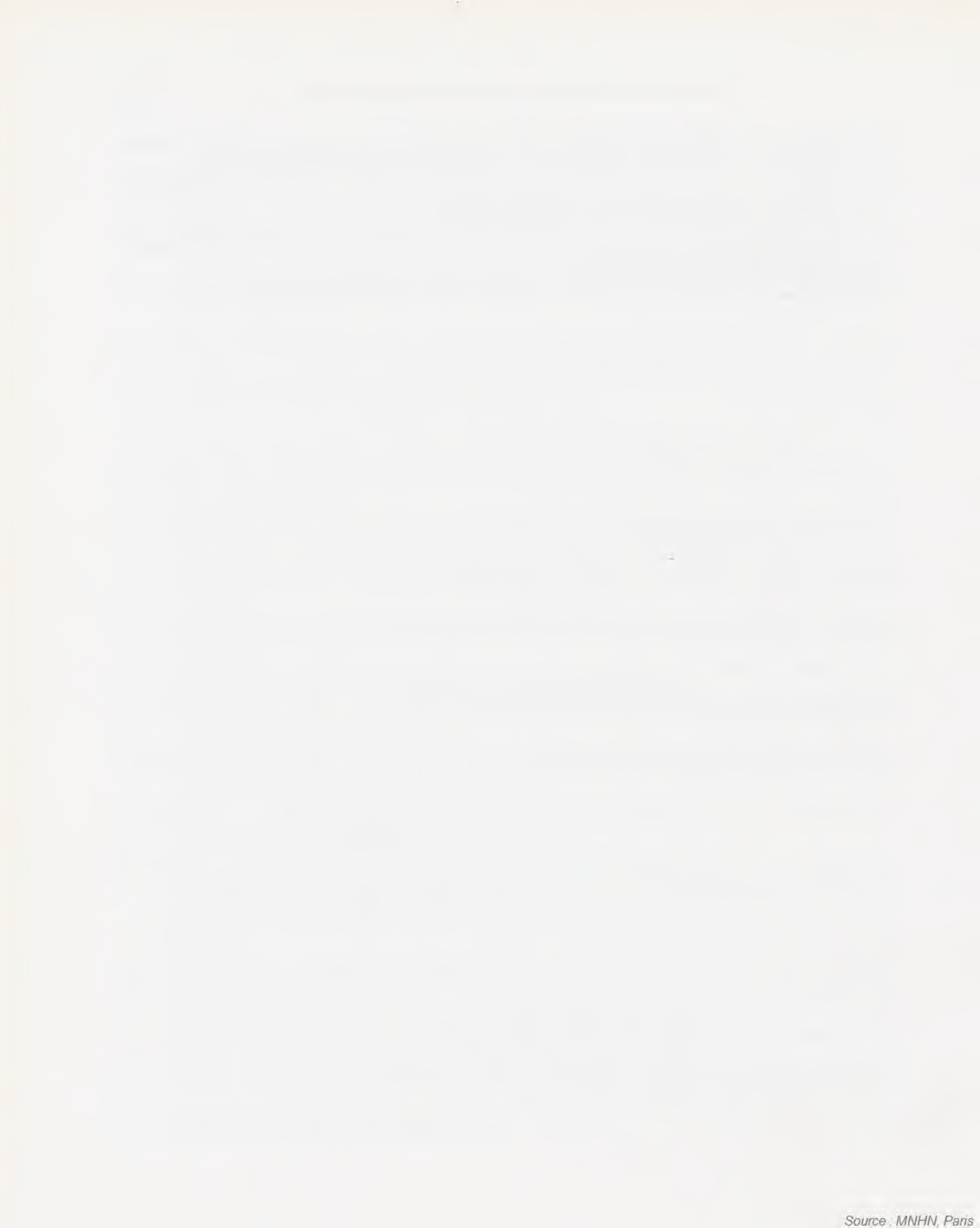
Source : MNHN, Paris



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Phylogeny of the Brachyura (Crustacea, Decapoda): Evidence from Spermatozoal Ultrastructure

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ABSTRACT

Spermatozoa of *Dynomene* aff. *devaneyi* (Dynomenidae) and *Homolodromia kai* (Homolodromiidae) are described. Parsimony analyses affirm the classification of the Brachyura by GUINOT (1978), notably the groupings Podotremata and Heterotremata *sensu lato*, as sister-groups, and Thoracotremata are confirmed. In the Podotremata, association of the Raninoidea and Cyclodorippoidea is upheld (as sister-groups), each with convincing and unique synapomorphies, but sperm data considered alone do not support alliance of the Homolidae, (a very clearly defined group) with this couplet and therefore do not endorse the grouping Archaeobrachyura which is, however, upheld by combined spermatozoal and non-spermatozoal data. The Dromiacea *sensu* Guinot (Dromiidae, Dynomenidae and Homolodromiidae) is confirmed spermatologically as a monophyletic grouping but the discreteness of the three constituent families is not upheld. *Homolodromia* displays a mixture of dromiid and dynomenid spermatozoal features. The Dynomenidae and Dromiidae are each found to be paraphyletic. *Latreillia* sp., considered an homoloid by GUINOT (1978) and GUINOT & RICHER DE FORGES (1995), forms a polytomy either with Homolidae+Raninoidea-Cyclodorippoidea with the combined, spermatozoal and non-spermatozoal, data set or with Homolidae+Dromiidae-Dynomenidae-Homolodromiidae, for sperm data only. The association by GUINOT (1978) of the Dorippoidea, Portunoidea, Xanthoidea, and Majoidea in the non-thoracotreme Heterotremata is fully supported spermatologically. Spermatozoal data give majids the most basal position in the Heterotremata whereas for the combined data *Neodorippe* (with carrying behaviour, like most podotremes) appears the least modified member of the heterotreme-thoracotreme assemblage. The Thoracotremata is unequivocally supported.

RÉSUMÉ

Phylogénie des Brachyura (Crustacea, Decapoda): le témoignage de l'ultrastructure des spermatozoïdes

Les spermatozoïdes de *Dynomene* aff. *devaneyi* (Dynomenidae) et *Homolodromia kai* (Homolodromiidae) sont décrits. Les analyses de parcimonie confirment la classification des Brachyura par GUINOT (1978), particulièrement les groupements Podotremata et Heterotremata *sensu lato* comme groupes-frères, et les Thoracotremata sont confirmés. Chez

JAMIESON, B. G. M., GUINOT, D. & RICHER DE FORGES, B., 1995. — Phylogeny of the Brachyura (Crustacea, Decapoda): evidence from spermatozoal ultrastructure. *In*: JAMIESON, B. G. M., AUSIO, J., & JUSTINE, J.-L. (eds), Advances in Spermatozoal Phylogeny and Taxonomy. *Mém. Mus. natn. Hist. nat.*, **166**: 265-283. Paris ISBN : 2-85653-225-X.

Source : MNHN, Paris

les Podotremata, l'association des Raninoidea et des Cyclodorippoidea est maintenue (comme groupes-frères), chacun avec des synapomorphies originales et convaincantes, mais les données spermatologiques utilisées seules ne permettent pas d'affirmer les relations des Homolidae (groupe très clairement défini) avec ces deux taxons, et donc ne supportent pas le groupement des Archaeobrachyura. Ce dernier est toutefois maintenu si l'on utilise à la fois les données spermatologiques et non spermatologiques. Les Dromiacea *sensu* Guinot (Dromiidae, Dynomenidae et Homolodromiidae) sont confirmés par les données spermatologiques comme un groupe monophylétique, mais le caractère séparé des trois familles n'est pas prouvé. *Homolodromia* montre un mélange de caractères spermatologiques de Dromiidae et de Dynomenidae. Les Dynomenidae et les Dromiidae ont tous deux été trouvés paraphylétiques. *Latreillia* sp., considéré comme un Homoloidea par GUINOT (1978) et GUINOT & RICHER DE FORGES (1995), forme une polytomie ou bien avec les Homolidae+Raninoidea-Cyclodorippoidea si on utilise les données spermatologiques et non-spermatologiques combinées, ou avec les Homolidae+Dromiidae-Dynomenidae-Homolodromiidae en utilisant les données spermatologiques seules. L'association par GUINOT (1978) des Dorippoidea, Portunoidea, Xanthoidea et Majoidea dans les Heterotremata non-thoracotrèmes est parfaitement confirmée par la spermatologie. Les données spermatologiques donnent aux Majidae la position la plus basale dans les Heterotremata alors que, avec les données combinées, *Neodorippe* (un 'porteur', comme la plupart des Podotremata) apparaît le membre le moins évolué de l'assemblage Hétérotrèmes-Thoracotrèmes. Les Thoracotremata sont confirmés de manière non équivoque.

The literature on sperm ultrastructure in Crustacea, and its relevance to phylogeny, a subject briefly addressed earlier for the Brachyura by BROWN [2], has been reviewed by JAMIESON [18]. Several papers on brachyuran ultrastructure have since appeared [19, 20, 23-27] and have culminated in a cladistic, parsimony analysis of brachyuran phylogeny [21] which is extended in the present chapter. The analyses apply the principles of phylogenetic systematics propounded by HENNIG [13] and computer procedures for phylogenetic analysis under the principle of parsimony which are enunciated by SWOFFORD [32].

The internal relationships and classification of brachyuran crabs, and particularly of the Podotremata, have been the subject of controversy. GUINOT [4-8] divides the Brachyura into three sections mainly on the basis of the location of the male and female pores: the Podotremata, the Heterotremata and the Thoracotremata. Nevertheless, GUINOT ([5]: p. 218) recognized that the coxal positions of male and female pores, with external fertilisation, characterizing the podotremes, were symplesiomorphies.

The Podotremata *sensu* GUINOT contain the Dromiacea and Archaeobrachyura. The Dromiacea consist of the Dromioidea and Homolodromioidea. The Archaeobrachyura contain the Homoloidea, Raninoidea, and Cyclodorippoidea (= Tymoloidea). In other classifications the superfamily Homoloidea, which includes three families (Homolidae, Latreilliidae and Poupiniidae) is often associated with or placed in the Dromiacea (see [5, 6, 12]).

The Heterotremata and Thoracotremata share a sternal location of the female pores and development of a sternal vulva on sternite 6, in direct communication with the seminal receptacle, allowing for internal fertilization. The Thoracotremata differ in the additional sternal location of the male pores. Whereas the Thoracotremata appeared to be a monophyletic group, the Heterotremata were suspected by JAMIESON to be paraphyletic [18].

In some contrast with the classification of GUINOT, nucleotide sequences of 18S ribosomal RNA support the exclusion of a mono- or poly-phyletic Dromiidae from the Brachyura, and their association with the Anomura, but support inclusion of the Raninidae in the Brachyura [1, 30, 31]; homolids were not considered in the molecular analyses.

This chapter adds to the former data matrix [21] new spermatozoal data on two families of questionable relationships, the Dynomenidae, represented by *Dynomene* aff. *devaneyi*, and the Homolodromiidae, represented by *Homolodromia kai*. The augmented matrix is subjected to parsimony analysis. In a second analysis, a minimum of non-spermatozoal characters, defining the Podotremata, Heterotremata and Thoracotremata and separating these from the Anomura, is added and effects on the original phylogram observed, pending a more comprehensive inclusion of non-spermatozoal characters.

Source : MNHN, Paris

MATERIAL AND METHODS

The species examined and sources of material are listed by JAMIESON [21]. In addition, the material of *Dynomene* aff. *devaneyi* and *Homolodromia kai* was obtained on the BATHUS 3 cruise in New Caledonian waters, at stations CP 805 and CC 848 respectively, on 22 November 1993.

Electron microscopy. Transmission electron microscopy procedures were as in [27].

Cladistics. Methods employed in the parsimony analysis are given in [21]. Characters employed are given in Table 1 and the data matrix is shown in Table 2. The parameters and specifications for the phylograms obtained are given in the legends of Fig. 1A and B.

TABLE 1. — Character coding employed

Spermatozoal characters

(1) Acrosome length:width: 0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0 1.1 1.2 1.3 1.4 1.5 1.6 1.7 1.8 1.9 2.0,
(2) Zonation of the contents of the acrosome vesicle predominantly: horizontal 0, concentric 1, intermediate 2,
(3) Operculum: imperforate 0, perforate, open 1, perforate, closed with apical button 2,
(4) Opercular projections into subopercular material absent 0, present 1,
(5) Operculum: discontinuous with capsule 0, continuous with capsule 1,
(6) Operculum: moderately thick 0, very thin double lamina 1,
(7) Operculum width: not extremely wide 0, extremely wide 1,
(8) Periopercular rim: absent 0, weak 1, well developed 2,
(9) Accessory opercular ring: absent 0, present 1,
(10) Subopercular protuberance through operculum: absent 0, weak 1, well developed 2,
(11) True acrosome ray zone: absent 0, present 1, lost 2,
(12) Outer acrosome zone border with peripheral zone: not ragged 0, ragged 1,
(13) Anterolateral pale zone of acrosome contents: absent 0, present 1,
(14) Flangelike peripheral extension of lower acrosome zone: absent 0, present 1,
(15) Xanthid ring: absent 0, present 1, modified and short 2, modified and elongate 3,
(16) Subacrosomal chamber or perforatorium: postequatorial 0, extending preequatorially 1,
(17) Head of perforatorium: non-capitate 0, amoeboid 1, spiked wheel 2, bilateral 3,
(18) Corrugations of wall of perforatorial chamber: absent 0, simple invaginations 1, branched invaginations 2, invaginations with filaments 3, filaments only 4, evaginations only 5,
(19) Lateral arms: absent 0, one 1 (not found), two 2, three 3, several 4
(20) Lateral arms: absent 0, microtubular with chromatin 1, nuclear only 2, microtubular only 3,
(21) Centrioles: absent 0, present 1, elongate 2. (Excluded).
(22) Posterior median process of nucleus: absent 0, present 1,
(23) Thickened ring: absent 0, present 1,
(24) Concentric lamellae: absent 0, present 1,
(25) Capsular chambers: absent 0, one chamber 1, several 2,
(26) Capsular projections: absent 0, present 1,
(27) Capsular flange: absent 0, present 1,

Non-spermatozoal characters

(28) Genital pores: all coxal 0, female sternal 1, male and female sternal 2,
(29) Separate spermatheca: absent 0, present 1,
(30) P5, reduction of: absent 0, present 1,
(31) P5, dorsal or subdorsal origin: absent 0, present 1,
(32) P5, subcheliform or cheliform modification: absent 0, weak 1, strong 2,
(33) Sella turcica: absent 0, present 1,
(34) Uropods: present 0, vestigial 1, absent 2

In the present analyses, characters were unordered excepting 1, 8, 11, 25 and 32 (ordered) and 34 (irreversible, up).

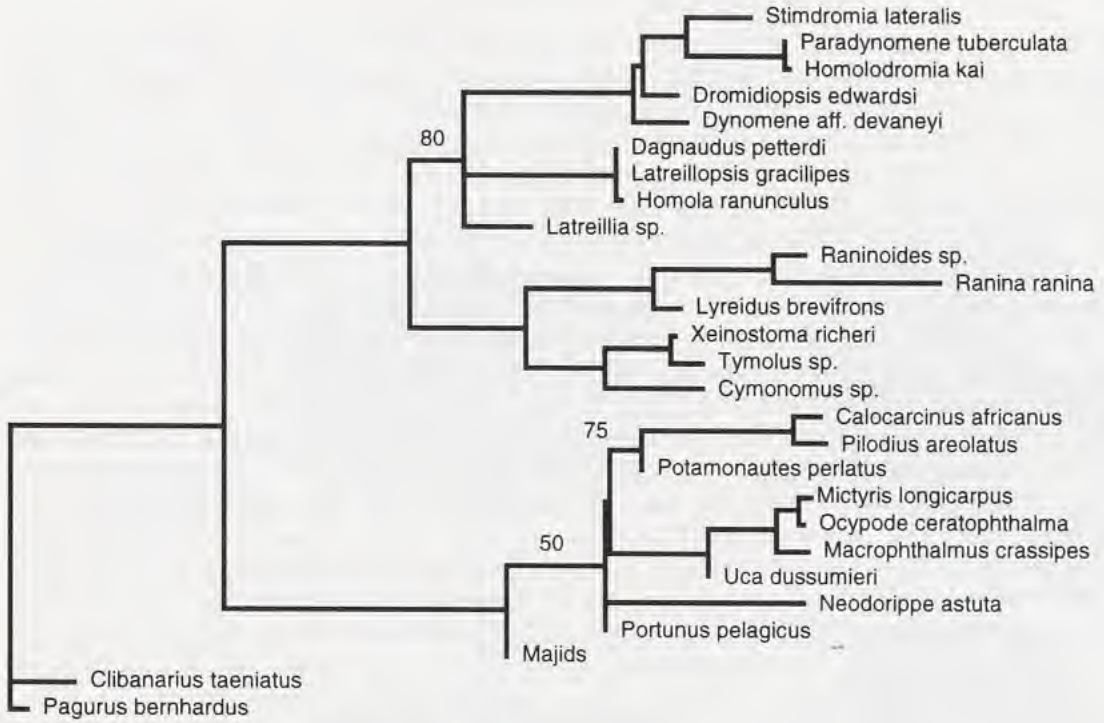
Source : MNHN, Paris

TABLE 2. — Data matrix

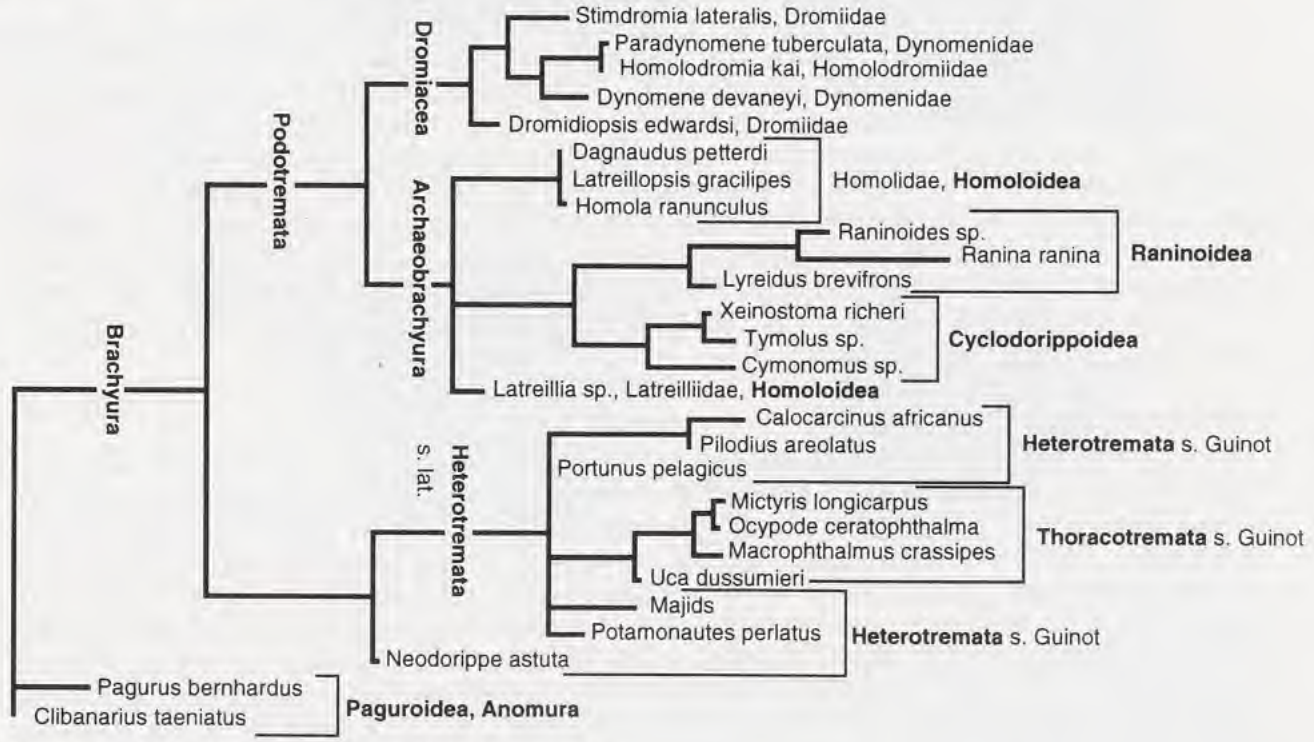
Taxon	111111111122222222233333 123456789012345678901234567890123 4
Stimdromia lateralis	30100000020010013000000000100111211
Dagnaudus petterdi	60110000010000012032110000000111212
Calocarcinus africanus	81000002101100210042101000010000 12
Dromidiopsis edwardsi	32100000020010013032000000000111211
Paradynomene tuberculata	30100000020011013000?0000000111111
Latreillopsis gracilipes	60110000010000012032110000000111212
Raninoides sp.	72101000000000001023201002110111012
Lyreidus brevifrons	52101000000000001113201000100111012
Xeinostoma richeri	50100110000000001033211000100111112
Cymonomus sp.	60000010000001010332?1000100111112
Tymolus sp.	60100110000000001033211000100111212 1
Neodorippe 'astuta'	B1000000001000010042001000010112 12
Portunus pelagicus	A1000000001000010042101000010000 12
Mictyris longicarpus	C1200000002000310042001100020000 12
Ocypode ceratophthalmus	9120000000200031004200110002000012
Uca dussumieri	9120000000200001004200100002000012
Macrophthalmus crassipes	A1000000002000010042001100020000 12
Pilodius areolatus	9100000010110011004200100001000012
Ranina ranina	81101000000000000232110011101010 12
Homola ranunculus	50110000010000012032?1000000111212
Majids	A1100000001000010 0?111100001000012 2
Potamonautes perlatus	9100000200100001004220100001000012
Latreillia sp.	62100000000000130?2?1000000111212
Pagurus bernhardus	F1000000000000010431110000000 10200
Clibanarius taeniatus	C1000000000000001043100000000010200
Homolodromia kai	40100000020011013000 0?000000111111
Dynomene aff. *devaneyi*	50100000020010013022000000000111111

FIG 1. — Trees of the Brachyura. **A:** Heuristic 50% Majority rule consensus tree of 959 shortest and equally parsimonious trees for spermatozoal characters only. Heuristic search settings: Addition sequence: simple. One tree(s) held at each step during stepwise addition. Tree-bisection-reconnection (TBR) branch-swapping performed. MULPARS option in effect. Steepest descent option not in effect. Branches having maximum length zero collapsed to yield polytomies. Topological constraints not enforced. Trees rooted by outgroup. Multi-state taxa interpreted as polymorphism. Character 21 excluded. Character-state optimization: Accelerated transformation (ACCTRAN). Tree length = 49771. Consistency index (CI) = 0.665. Homoplasy index (HI) = 0.352. CI excluding uninformative characters = 0.647. HI excluding uninformative characters = 0.359. Retention index (RI) = 0.885. Rescaled consistency index (RC) = 0.588. Clades are supported by 100% of trees unless otherwise indicated. **B:** Heuristic strict consensus tree of 36 shortest and equally parsimonious trees, for spermatozoal and non-spermatozoal characters, using the outgroup method. Setting as for (A). Tree length = 47210. Consistency index (CI) = 0.701. Homoplasy index (HI) = 0.317. CI excluding uninformative characters = 0.682. HI excluding uninformative characters = 0.324. Retention index (RI) = 0.902. Rescaled consistency index (RC) = 0.632.

Source : MNHN, Paris



A Spermatozoal characters only



B Spermatozoal and non-spermatozoal characters

Source : MNHN, Paris

RESULTS AND DISCUSSION

In the parsimony analysis of spermatozoal data, the heuristic search option was used as computations under the branch and bound option were not completed in reasonable time. Nevertheless, the resultant phylograms agreed closely with branch and bound trees previously obtained [21]. The combined, spermatozoal and non-spermatozoal data yielded a highly structured strict consensus tree (Fig. 1B). Spermatozoal data alone gave an unstructured, completely pectinate strict consensus tree but the 50% Majority Rule consensus tree (Fig. 1A) was highly dichotomous and clearly meaningful, despite criticisms which have been levelled at the validity of majority consensus, in terms of resultant groupings, notably the dromiaceans, homolids, raninoids, cyclodorippoids, heterotremes *sensu lato*, and thoracotremes, which are supportable on other grounds. Conclusions from the two consensus trees are discussed below. Non-spermatozoal characters will be discussed only where especially relevant but have had more extensive treatment in the previous analysis [21].

The chief difference between the two trees is that the Homolidae and Latreillidae are associated with the Raninoidea+Cyclodorippoidea in the anlaysis of combined, spermatozoal and non-spermatozoal data (hereafter termed the combined analysis) (Fig. 1B), but associate with the Dromiacea in the purely spermatozoal analysis (Fig. 1A). The former assemblage corresponds with and supports the recognition of a taxon Archaeobrachyura by GUINOT [5]. Discussion of the succession of spermatozoal apomorphies and of group synapomorphies in the following account will chiefly be derived from the combined analysis but, with the exception noted and some others to be discussed, there is strong agreement between the two analyses. It is stressed that a larger and more refined suite of morphological characters is required for a combined analysis (GUINOT *et al.*, in preparation).

Brachyura

The Brachyura is a monophyletic taxon relative to the anomuran outgroup, *Pagurus bernhardus* and *Clibanarius taeniatus*. Although the sperm of the Anomura [34] and Brachyura are distinctive relative to other decapods, the Brachyura have only weak spermatozoal synapomorphies relative to anomurans despite forming a monophyletic brachyuran clade. Brachyuran monophyly is supported by shortening of the acrosome to a nearly spheroidal form; loss of corrugations of the wall of the perforatorial chamber, though these reappear in a different form in raninoids and cyclodorippoids; loss of microtubules from the lateral arms, a doubtful synapomorphy in view of their presence in at least some majids [14]; and, somatically, development of a sella turcica and reduction of the uropods. Although spermatozoal support for a monophyletic Brachyura is weak, many constituent groups are, in contrast, strongly supported.

Podotremata

In both the combined and the solely spermatozoal analysis, the Podotremata is a monophyletic taxon and the sister-group of the heterotreme-thoracotreme assemblage (Fig. 1A, B), as also shown previously [11, 21]. Synapomorphies of podotreme spermatozoa, as indicated in the combined anlaysis, include depression of the acrosome; development of a predominantly horizontal zonation of the acrosome compared with the concentric zonation of paguroids and heterotremes; and (ambiguously) a bilaterally symmetrical capitate perforatorial head (developing from the simple, non-capitate form in paguroids and ancestral crabs), which is lost in some members. The bilateral perforatorial head is seen in dromiids (*Dromidiopsis edwardsi* and *Stimdromia lateralis*); in the two investigated dynomenids (*Paradynomene tuberculata*, [21], and *Dynomene* aff. *devaneyi*) and in *Homolodromia kai* and contrasts with that of homolid sperm which has the form of a horizontally disposed spiked wheel [21, 27].

Source : MNHN, Paris

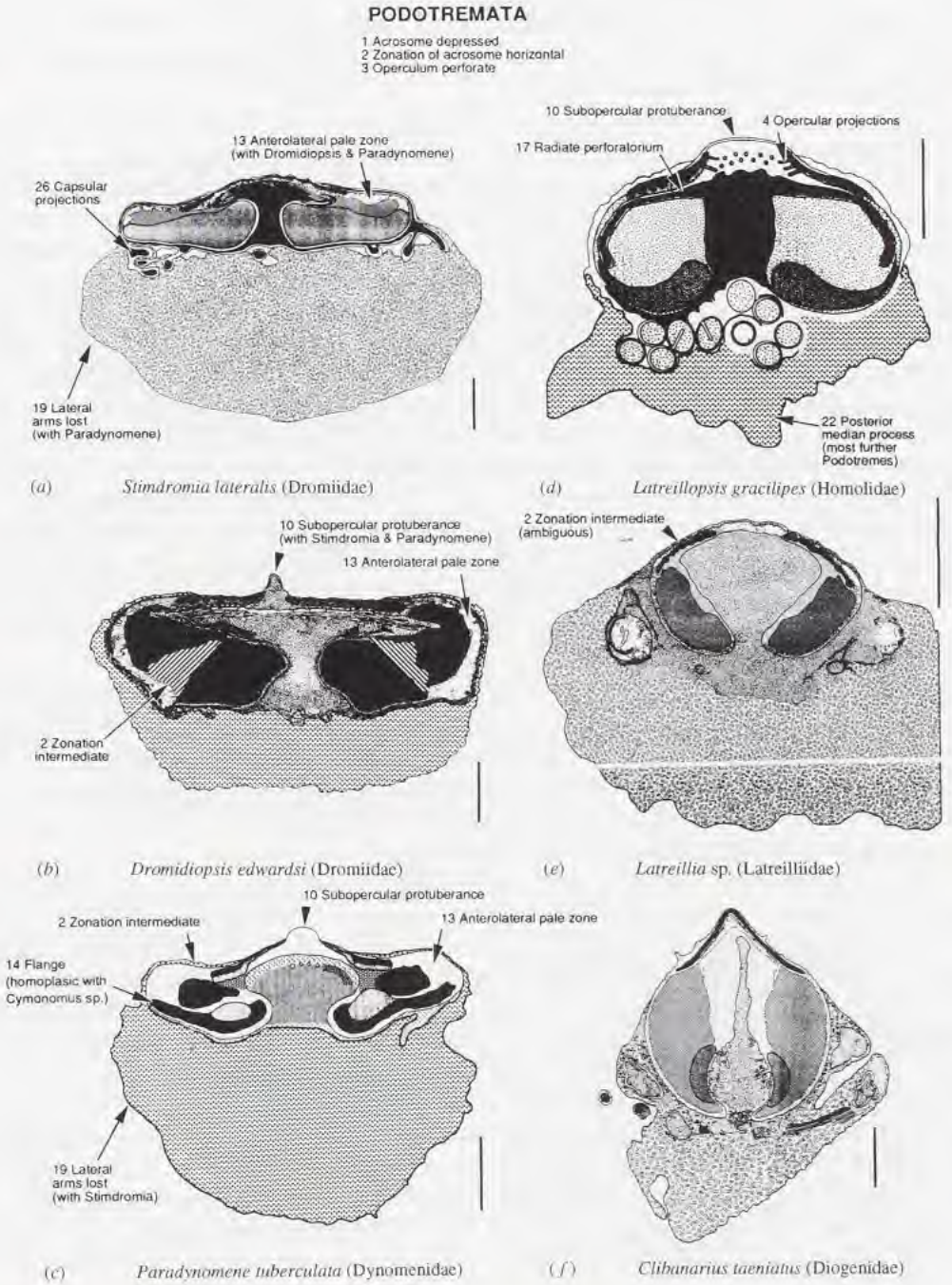


FIG. 2. — Drawings of spermatozoa of some podotremes and an anomuran used in this analysis. **a:** *Stimdromia lateralis* (Dromiidae). **b:** *Dromidiopsis edwardsi* (Dromiidae). **c:** *Paradynomene tuberculata* (Dynomenidae). **d:** *Latreillopsis gracilipes* (Homolidae). **e:** *Latreillia* sp. (Latreilliidae). **f:** *Clibanarius taeniatus* (Anomura, Diogenidae). The chief apomorphies are indicated but see text for a more detailed explanation. The section of *Stimdromia* (first described as *Petalomera* [17]) is not precisely sagittal; in micrographs which are sagittal, perforation of the operculum is seen. Scale bar 1 µm. After [21].

Source : MNHN, Paris

Apical perforation of the spermatozoal operculum is a further synapomorphy of podotremes, the same condition in majids being, it appears, independently derived (homoplasic). Monophyly of the Podotremata as deduced from species examined for sperm ultrastructure to date, does not exclude, nor does it support, the possibility that some supposed dromiids, notably *Hypoconcha* [31], have been missclassified and may be closer phylogenetically to anomurans that they are to other brachyurans.

Dromiacea. The Dromiacea as constituted by GUINOT for the Dromiidae, Homolodromiidae, and Dynomenidae [5, 10], is confirmed as a monophyletic group in both analyses (Fig. 1A, B). Its spermatozoal synapomorphies, from the combined analysis, are further depression of the acrosome, well developed protrusion of subopercular material through the operculum (a lesser protrusion occurs in homolids), and development of an anterolateral pale zone of the acrosome. Although the Dromiacea forms a monophyletic clade, neither the constituent Dromiidae nor the Dynomenidae appears monophyletic spermatologically. Thus, in the combined analysis (Fig. 1B) *Paradynomene* pairs with *Homolodromia*, and these have *Dynomene* as their sister-group, the three being closer to *Stimdromia* than this is to the other dromiid, *Dromidiopsis,* which forms the sister-group of the other dromiaceans. In the purely spermatozoal analysis (Fig. 1A), *Paradynomene* again pairs with *Homolodromia* but sister-groups, in descending order, are *Stimdromia*, *Dromidiopsis* and *Dynomene*. It can thus be said that although there is distinctive dromiacean spermatozoal ground plan, sperm structure does not distinguish the constituent families Dromiidae, Homolodromiidae and Dynomenidae. This does not necessarily challenge definition of these families on the grounds of non-spermatozoal morphology (e.g. [10, 29]) and further analysis of non-spermatozoal characters is in progress to further ascertain the relationships of these families (GUINOT, JAMIESON & RICHER DE FORGES, and GUINOT & TAVARES, in preparation).

Dromiidae. The Dromiidae (see [29]) are elusive of definition spermatologically as shown in the previous section (see also [21]), being a paraphyletic group in both analyses. In the combined analysis (Fig. 1B), a monophyletic dromiid clade (including dynomenids and *Homolodromia*) is identical with the dromiacean clade. Spermatozoa of *Stimdromia (=Petalomera) lateralis*, *Dromidiopsis edwardsi* and *Paradynomene tuberculata* are illustrated in Fig. 2A-C and that of *Homolodromia kai* in Fig. 6B.

In the combined analysis (Fig. 1B), *Dromidiopsis edwardsi* is the sister-taxon of the other dromiaceans. The sole, and somewhat subjective, apomorphy of the sperm of *Dromidiopsis edwardsi* [28] is a zonation of the acrosome which is intermediate between the horizontal and concentric conditions. Synapomorphies of the dromiid-dynomenid-*Homolodromia* melange are weak, being loss of the three arms basic to the anomuran-brachyuran assemblage, and with them any microtubules in these arms. As arms are present in *Dynomene* aff. *devaneyi*, their basal loss is questionable, but they may well be labile in occurrence. *Stimdromia lateralis* (Fig. 2A) is diagnosed by the presence of capsular projections. *Dynomene* aff. *devaneyi*, which computes as basal relative to these taxa, appears to be unique in the Brachyura, in having only two nuclear arms. A further apomorphy is slight lengthening of the acrosome. *Paradynomene* (Fig. 2C) and *Homolodromia* (Fig. 6B) have a striking similarity, computing as a synapomorphy: a flange like lateral extension of the lower acrosome zone. *Paradynomene* is distinguished (ambiguously) by slight lengthening of the acrosome whereas *Homolodromia* shows no individual apomorphy; in the spermatozoal anlaysis, it is distinguished from *Paradynomene* only by its slightly more depressed acrosome.

Centrioles are unknown in dromiid sperm but are present in homolids. The difficulty in unequivocally demonstrating their presence or absence has led to their exclusion from the parsimony analyses.

Homolodromiidae This family is placed in a monotypic superfamily Homolodromioidea,

Source : MNHN, Paris

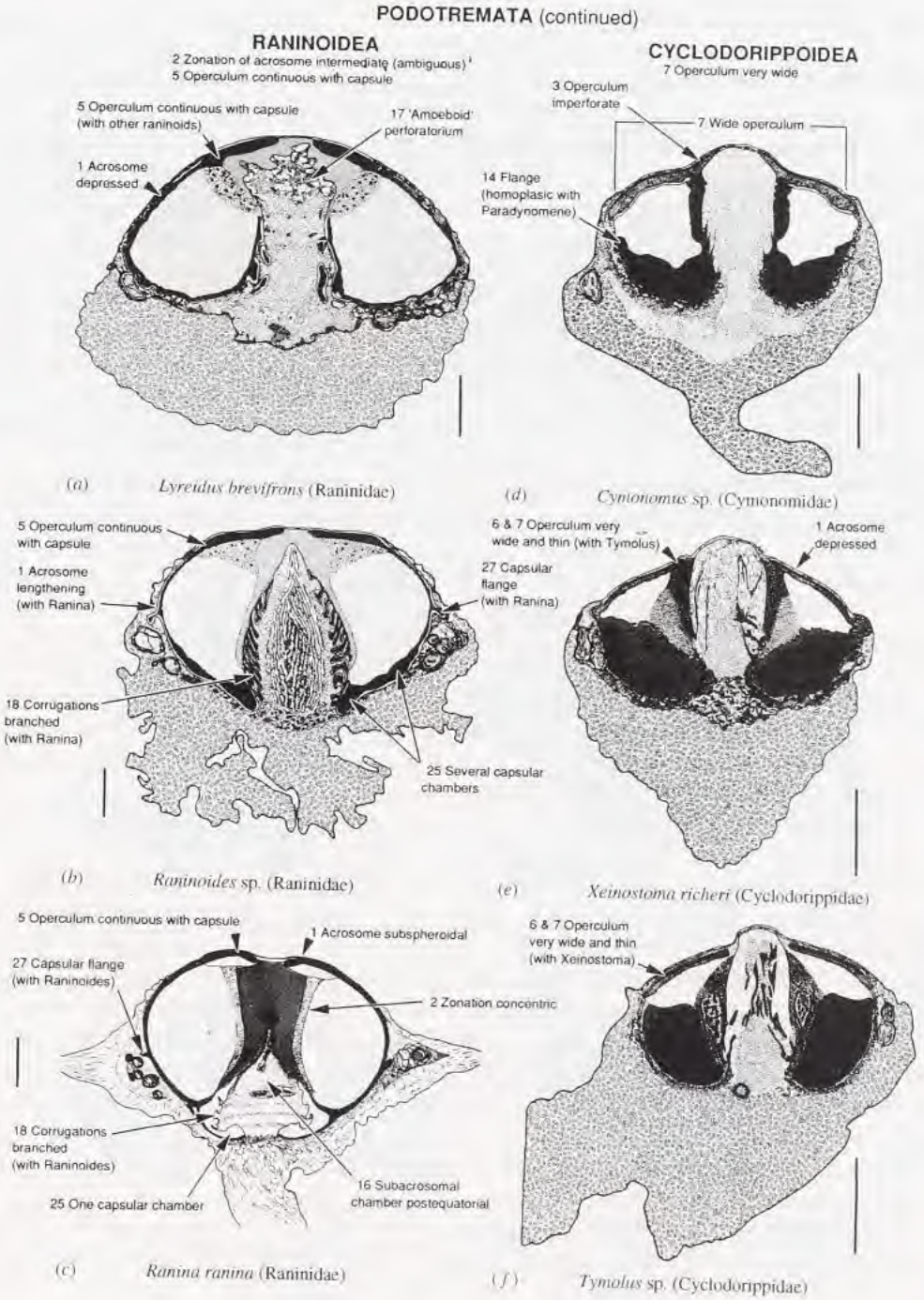


FIG. 3. — Drawings of spermatozoa of further podotremes used in this analysis. **a:** *Lyreidus brevifrons* (Raninidae, Lyreidinae). **b:** *Raninoides* sp. (Raninidae, Raninoidinae). **c:** *Ranina ranina* (Raninidae, Raninninae). **d:** *Cymonomus* sp. (Cymonomidae). **e:** *Xeinostoma richeri* (Cyclodorippidae, Xeinostominae). **f:** *Tymolus* sp. (Cyclodorippidae, Cyclodorippinae). The chief apomorphies are indicated but see text for a more detailed explanation. Scale bar 1 µm. Sources as listed in Material and methods. After JAMIESON [21].

Source : MNHN, Paris

within the Dromiacea, by GUINOT [5, 10]. She considers that the Homolodromioidea represent, without doubt, the most primitive [members] of the Podotremata and lists a long series of characters in support of this contention. It is difficult, therefore, to evaluate the relatively advanced position which *Homolodromia* appears to occupy, in terms of spermatozoal ultrastructure, relative to other dromiaceans (Fig. 1A, B). It is noteworthy, in view of origin of *Homolodromia* in the phylograms between *Paradynomene* on the one hand and *Dynomene*, with or without intervention of dromiids, that GUINOT [5] stated that in some regards it is the dynomenids which seem closer to the Homolodromiidae than do the Dromiidae. The fact that *Homolodromia* lies within a dromiid clade is also of interest with regard to GUINOT'S [5, 10] statement (drawing on [35] and others) that the level of organization of the fossil Prosopidae, the most ancient crabs known, survives on the one hand in the form (without doubt little modified) of the Homolodromioidea, which inhabit deep waters, and on the other hand in the form of the Dromioidea (Dromiidae and Dynomenidae), much more numerous and diversified, which have developed special adaptations (in most Dromiidae the carapace is protected by a sponge, an ascidian or a bivalve shell) [29]. The Homolodromiidae have a unique combinaton of morphological characters, though mostly plesiomorphic. These are, *inter alia*, fusion of the ophthalmic segment to the anterior carapace (in *Homolodromia*); the soft branchiostegite; endophragmal skeleton with anastomoses; abdominal pleura developed; and retention of abdominal pleopods in the male on segments 3 to 5. Occurrence of uropods which are not dorsal and are represented by small lobes on the abdominal segment 6 appears to be a homolodromiid synapomorphy [10]. The phylograms (Fig. 1A, B) are heuristic for reconsideration of the validity and relationships of the families Dromiidae, Homolodromiidae and Dynomenidae.

In terms of the ultrastructural characters used in the parsimony analyses, the spermatozoon of *Homolodromia kai* has the following characteristics. The ratio of length to width of the acrosome is 0.4; zonation of the acrosome is predominantly horizontal; the operculum is perforate and lacks opercular projections such as are diagnostic of homolids; the operculum is not continuous with the acrosomal capsule, and, in contrast with raninoids, it is moderately thick and is of moderate width, not thin and occupying much of the width of the acrosome as in cyclodorippoids; there is no peripercular rim nor an accessory opercular ring; protrusion of subopercular material through the operculum is well developed; a true acrosome ray zone of the type seen in paguroids, other anomurans and in brachyurans of the Heterotremata *sensu stricto*, is absent although a "finger-print" like zone is possibly homologous with this; the ragged outer acrosomal zone and the xanthid ring, typical of xanthids and some of their relatives, are absent; an anterior pale zone of the acrosome, seen also in *Stimdromia*, *Dromidiopsis*, *Dynomene* and *Paradynomene*, is present; the subacrosomal chamber extends pre-equatorially in the acrosome as in all investigated species excepting *Ranina ranina*; the head of the putative perforatorium is bilaterally symmetrical, as in *Stimdromia*, *Dromidiopsis* and *Paradynomene*; corrugations of the wall of the perforatorial chamber, a thickened ring, concentric lamellae, capsular chambers, projections and flanges are absent. Nuclear arms and a definite posterior median process are not demonstrable.

Dynomenidae. GUINOT [5, 8, 10], and GUINOT, JAMIESON & RICHER DE FORGES [11], ranked dynomenids as a family in the superfamily Dromioidea, placed with the Homolodromioidea in the subsection Dromiacea, within the section Podotremata. This placement of dynomenids is wholly supported in both analyses but as indicated above, the Dynomenidae does not have spermatological support as a monophyletic group (Fig. 1A, B).

Separation of the Dynomenidae from the Dromiidae is justified, in non-spermatozoal characters, by a large number of differences [6] which include complete modification of the coxa of P5 as a penis. Furthermore, dynomenids show reduction of P5 instead of P4 and P5 as in the

Source : MNHN, Paris

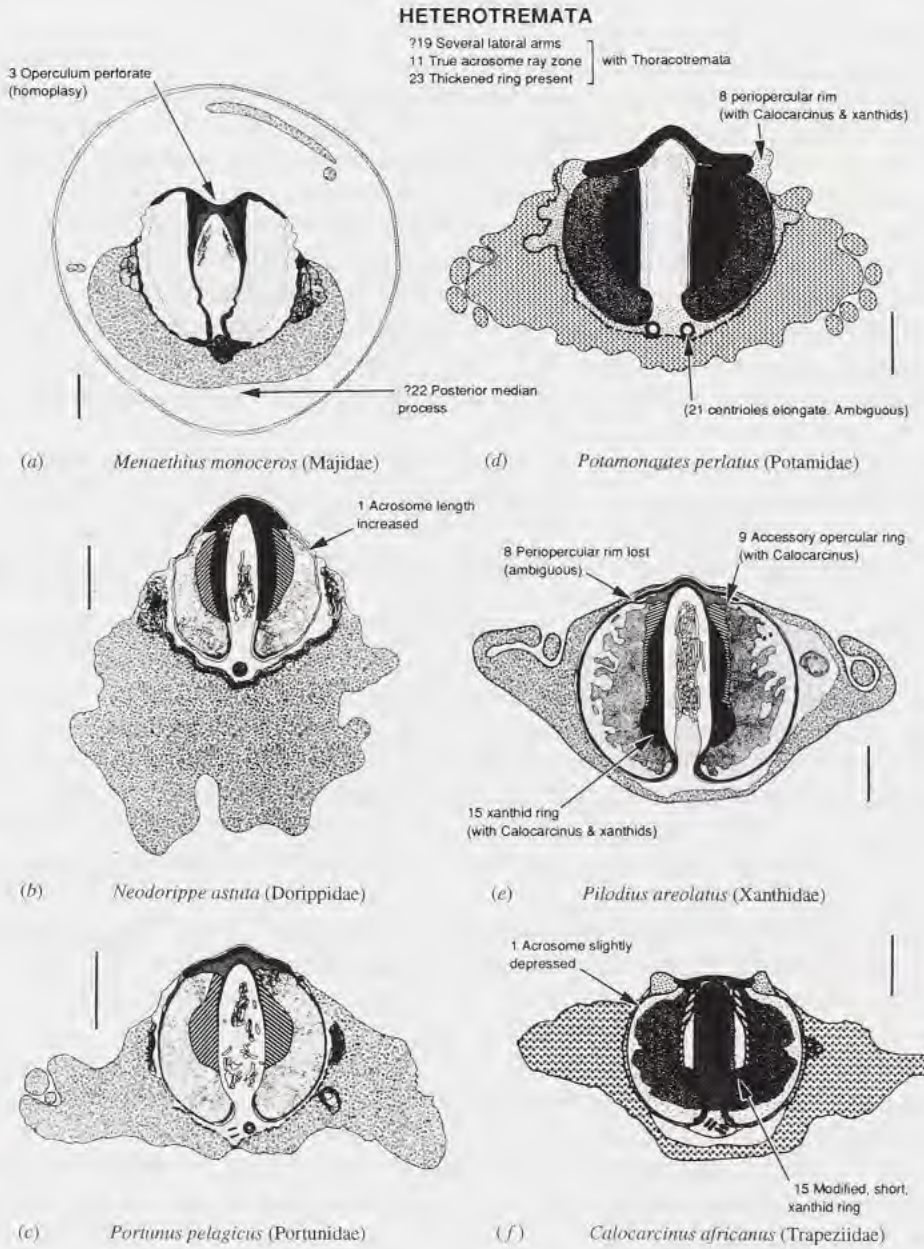


FIG. 4. — Drawings of spermatozoa of Heterotremata used in this analysis. **a:** *Menaethius monoceros* (Majidae). **b:** *Neodorippe 'astuta'*, now considered close to *N. callida* (Dorippidae). **c:** *Portunus pelagicus* (Portunidae). **d:** *Potamonautes perlatus* (Potamidae). **e:** *Pilodius areolatus* (Xanthidae). **f:** *Calocarcinus africanus* (Trapeziidae). The chief apomorphies are indicated but see text for a more detailed explanation. Scale bar 1 µm. Sources as listed in Material and methods. After [21].

Source : MNHN, Paris

Dromiidae. Despite the more brachyuran facies of some species, several features of the Dynomenidae appear to be plesiomorphic and to accord with the earlier appearance of dynomenids in the fossil record relative to dromiids.

The sperm of *Dynomene* aff. *devaneyi* (Fig. 6A) resembles that of *Homolodromia kai*, described above, in all features mentioned, with the exception of the following. The ratio of length to width of the acrosome is 0.5; two nuclear arms are detectable; and a posteromedian process is absent.

Archaeobrachyura. The phylogram for combined data (Fig. 1B), as previously [21], supports recognition of the Archaeobrachyura of GUINOT [5], containing the superfamilies Homoloidea, Raninoidea and Cyclodorippoidea (=Tymoloidea). The single spermatozoal synapomorphy for the Archaeobrachyura is weak: the presence of a posterior median process. It is, however, reinforced by the somatic character loss of the uropods [21]. The grouping Archaeobrachyura is not, however, supported in the purely spermatozoal analysis (Fig. 1A) in which *Latreillia* and the homolids group with the Dromiacea (Dromiidae, Dynomenidae and Homolodromiidae) and not with the raninoid+cyclodorippoid assemblage.

Homolidae. Spermatozoal ultrastructure has been examined in seven species of the Homolidae: *Homola ranunculus*, *Paramola bathyalis* and *Dagnaudus (=Paramola) petterdi* [11, 12] and in *Homologenus* sp., *Latreillopsis gracilipes* (Fig. 2D), *Homolomannia sibogae*, and *Paromolopsis boasi* [27].

From spermatozoal ultrastructure, the Homolidae is a convincingly monophyletic entity in the combined and the spermatozoal analyses (Fig. 1A, B, and [21]). Synapomorphies of homolid spermatozoa are the following. The presence of numerous radial arranged extensions of the acrosomal operculum into the perforatorium has been established as an autapomorphy of the homolids [27] seen in no other brachyurans. Projection of subacrosomal material into the opercular perforation occurs but is weaker than the strong protrusion which is apparently independently developed in dromiaceans. Thirdly, the spiked-wheel form of the anterior expansion of the perforatorium is restricted to the Homolidae for which it is thus an autapomorphy. Whether a prexisting bilateral form of the head of perforatorium is a basic condition of all podotremes or the non-capitate condition is basic computes ambiguously. The radial spikes, approximately 12 in number, extend far laterally. They are supported by fibrous cores which radiate from the central core of the perforatorium. The spikes are much longer in *Latreillopsis gracilipes* (Fig. 2D) than in the other species, curving around the inner aspect of the vesicle almost to its base.

Raninoidea and Cyclodorippoidea. The Raninoidea (Fig. 3A-C) and Cyclodorippoidea (Fig. 3D-F) form a monophyletic (but unnamed) clade in both analyses (Fig. 1A, B, and [21]). Spermatozoal synapomorphies are not striking and two are ambiguous: reversal from a bilateral to a non-capitate condition of the perforatorium; and development of simple corrugations of the wall of the perforatorial chamber. Unambiguous are development of outward projections of the capsule (present study and [21]), seen homoplasically in *Stimdromia*; and, somatically, though confined to the Raninoidea, loss of the subcheliform development of pereiopods 5.

Raninoidea. Spermatozoal ultrastructure has been investigated in *Ranina ranina* [16] (Fig. 3C), in the subfamily Ranininae, *Raninoides* sp. [26] (Fig. 3B), in the subfamily Raninoidinae (reinstated by GUINOT [9]), and *Lyreidus brevifrons* Sakai, 1937 [26] (Fig. 3A), in the subfamily Lyreidinae [9]. These raninoids, as a group, are well defined spermatologically (Fig. 1A, B) by virtual continuity of the operculum with the capsule and alteration of the zonation of the acrosome vesicle to an intermediate condition, with development of a concentric condition in *Ranina ranina*. The intermediate condition is homoplasic with *Dromidiopsis* and *Latreillia*. Somatically raninoids have lost subcheliform modification of pereiopods 5 (this study and [21]), perhaps correlated with a burrowing or swimming habit.

Source : MNHN, Paris

Ranina [16] and *Raninoides* [26] share strong synapomorphies: development of posterior capsular chambers, one in *Ranina* (Fig. 3C) increasing to several in *Raninoides* (Fig. 3B); and the remarkable lateral flange on the capsule. An ambiguous change, not shown in some parsimony analyses [21], is development of branched septum-like corrugations of the wall of the perforatorial chamber from the unbranched form basal to the raninoid-cyclodorippoid clade and persistent in *Lyreidus*. There is also a strong trend towards a subspheroidal form of the acrosome, most developed in *Ranina* in which zonation becomes concentric; and in which the perforatorium, apparently secondarily, becomes only postequatorial. In *Lyreidus* (Fig. 3A), the acrosome becomes secondarily depressed; and the "amoeboid" form of the head of the perforatorium is seen as development of a capitate condition independently of that in dromiids and homolids (This study and [21]).

Cyclodorippoidea. The Cyclodorippoidea form the sister-group of the Raninoidea in both analyses (Fig. 1A, B). The sperm of the three cyclodorippoids (Fig. 3D-F) [25] are well defined by the extreme width of the operculum relative to the acrosome. As an ambiguous change, corrugations of the wall of the perforatorial chamber are invaginations with filaments. A synapomorphy of *Xeinostoma* (Fig. 3E) and *Tymolus* (Fig. 3F) is the extreme thinness of the operculum. *Xeinostoma* is apomorphic in further depression of the acrosome. *Cymonomus* (Fig. 3D) is apomorphic for all investigated podotremes in losing the opercular perforation. This supports erection of a separate family Cymonomidae [33]. It appears to have developed the flange-like extension of the lower acrosome zone independently of *Paradynomene* and *Homolodromia* but the similarity is striking and cyclodorippoid relationships require further investigation (This study and [21], GUINOT & TAVARES, in preparation).

Latreilliidae. The position of *Latreillia* sp. (Fig. 2E) is equivocal, as in the previous cladistic analyses [21]. It forms a polytomy either with Homolidae+Raninoidea-Cyclodorippoidea with the combined data set (Fig. 1B) or with Homolidae+Dromiidae-Dynomenidae-Homolodromiidae, for sperm only (Fig. 1A). This archaeobrachyuran status of *Latreillia* for the combined data is in accordance with placement of the Latreilliidae by GUINOT [5] near the Homolidae and contradicts the view of WRIGHT AND COLLINS (see [5]) that the accepted close relationship between the Homolidae and Latreilliidae is based on no more than a few primitive features. Confirmation of the ultrastructural characteristics of *Latreillia* sperm is desirable as many spermatozoa of this species used in the cladistic study appeared malformed. The sole detected apomorphy of *Latreillia* is development, homoplasically with *Dromidiopsis*, of an intermediate condition of the acrosome vesicle contents from the horizontally zoned condition. In the combined analysis this condition is an ambiguous apomorphy as it could alternatively be basal to the Podotremata but it is unequivocal in the purely spermatozoal analysis.

Heterotremata and Thoracotremata

In the cladistic analyses (present study and [21]) (Fig. 1A, B), it is seen that within the heterotreme-thoracotreme assemblage, the Thoracotremata (Fig. 5) is a monophyletic taxon whereas the Heterotremata *sensu stricto* (Fig. 4) is a paraphyletic grouping.

The combined Heterotremata-Thoracotremata, which may be termed the Heterotremata *sensu lato* [21], is defined by a convincing synapomorphy, presence of the thickened ring. Other spermatozoal synapomorphies, although unambiguous, are less convincing. Multiplication of lateral arms from three, common to paguroids and podotremes, to several is a trend rather than a diagnostic basal apomorphy as it results from polymorphism, there being three in at least some majids as in the leucosiid *Iliacantha subglobosa* [3]. Presence of a true acrosome ray zone appears to be a synapomorphy but is seen, apparently homoplasically, in paguroids.

Cladistically (present study and [21]), the Heterotremata *sensu lato* form a grouping whether or not non-spermatozoal characters are included but the sternal female pores constitute, as GUINOT [5, 6] suggested, their non-spermatozoal synapomorphy. In the combined analysis

Source : MNHN, Paris

(Fig. 1B) as previously [21], *Neodorippe* forms the plesiomorphic sister-group of all other included crabs. Its sole (ambiguous) spermatozoal apomorphy is very slight elongation of the acrosome beyond a spheroidal shape. It is noteworthy, in view of their relatively plesiomorphic spermatozoal ultrastructure, that dorippids exhibit carrying behaviour, like most dromiids, *Neodorippe callida* attaching to leaves. The dorippid included here, and referred to as *Neodorippe astuta* (see [22]), is close to *N. callida* but definitive identification has not been made. There are, however, no spermatozoal apomorphies distinguishing the remaining crabs of the Heterotremata *sensu lato* from *Neodorippe*, though somatic synapomorphies are loss of subcheliform development of pereiopods 5 (and also P4). On the basis of purely spermatozoal data, as in the former analysis [21], the Majidae occupy this basal position (Fig. 1A).

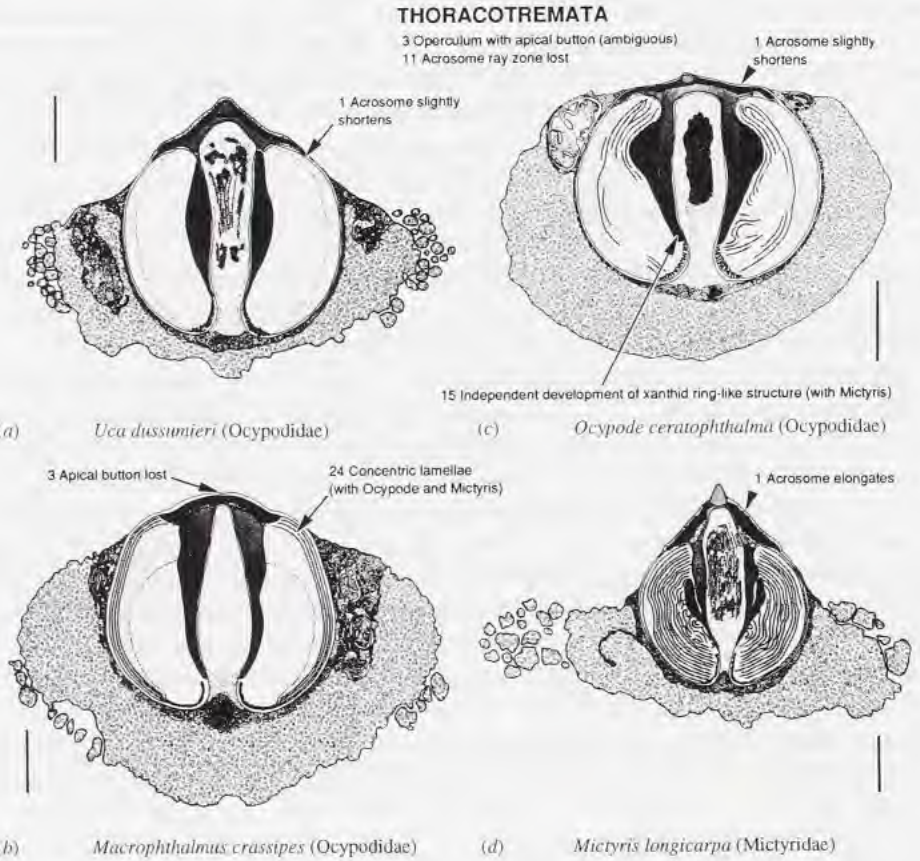
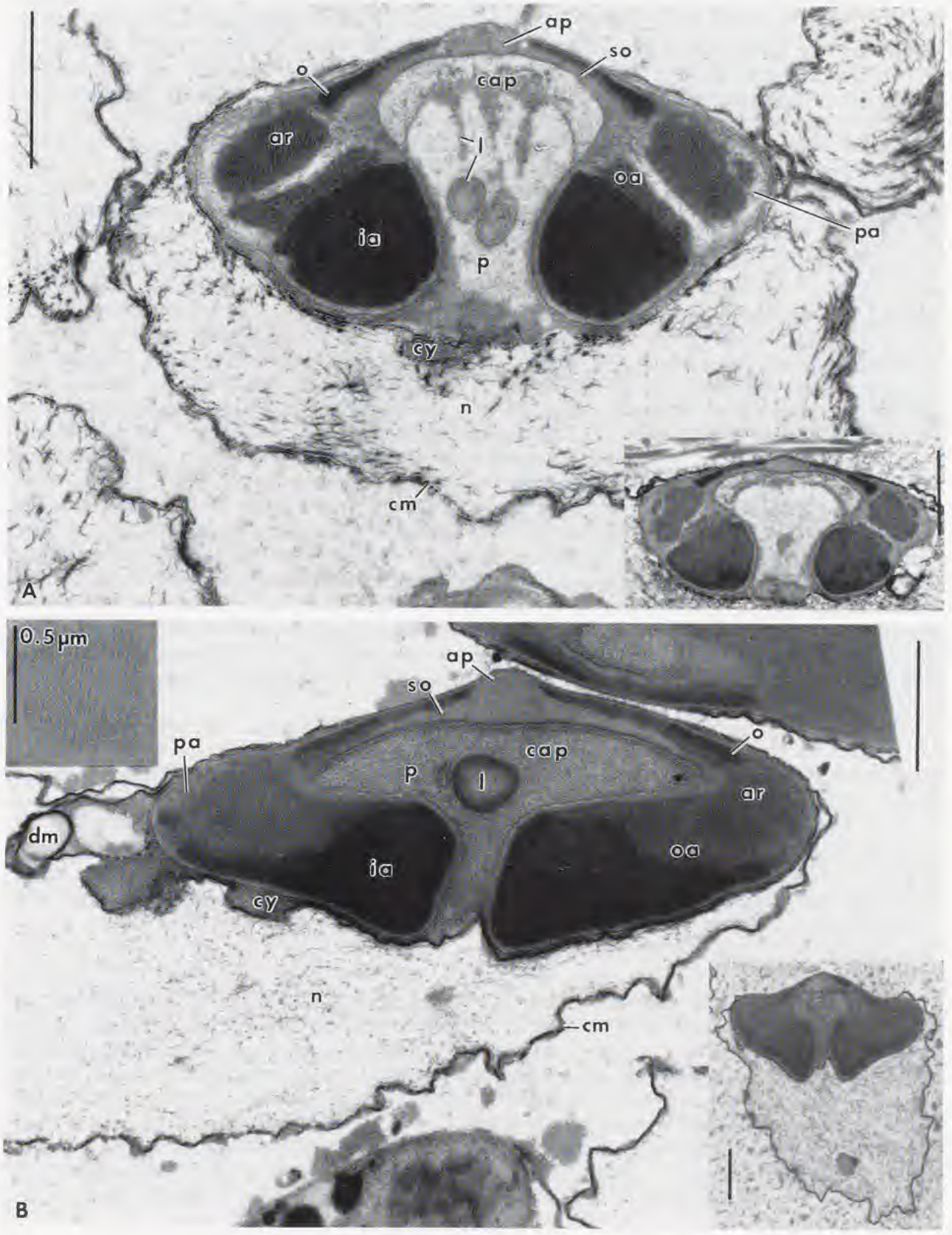


FIG. 5. — Drawings of spermatozoa of Thoracotremata used in this analysis. **a:** *Uca dussumieri*. **b:** *Macrophthalmus crassipes*. **c:** *Ocypode ceratophthalma* (all Ocypodidae). **d:** *Mictyris longicarpus* (Mictyridae). The chief apomorphies are indicated but see text for a more detailed explanation. Scale bar 1 µm. Sources as listed in Material and methods. After [21].

FIG. 6. — Transmission electron micrographs of longitudinal sagittal sections of the sperm of two podotreme species described in this chapter. **A:** *Dynomene* aff. *devaneyi*. Short diameter of perforatorium in main micrograph, long diameter right inset. **B:** *Homolodromia kai*. Long diameter of perforatorium in main micrograph, short diameter in right inset, detail of acrosome ray zone ("fingerprint" zone) in left inset. ap, apical protuberance; ar, acrosome ray zone; cap, capitate region of perforatorium; cm, cell membrane; cy, cytoplasm; dm, degenerating mitochondrion; ia, inner acrosome zone; l, lamellae; n, nucleus; o, operculum; oa, outer acrosome zone; p, perforatorium; pa, anterolateral pale acrosome zone; so, subopercular zone.

Source : MNHN, Paris



Source : MNHN, Paris

Within the heterotremes above *Neodorippe* (combined data, Fig. 1B), or above the majids (sperm only, Fig. 1A), *Calocarcinus* and the xanthid *Pilodius* group together but there is ambiguity as to whether development of a simple xanthid ring is basic to the two and is retained in xanthids but transformed in *Calocarcinus* (as seems likely), whether the supposedly transformed condition is basal, or whether each developed a form of the xanthid ring *de novo*. Their ancestor may have slightly shortened the acrosome. Other significant synapomorphies, retained in *Calocarcinus* and *Pilodius*, are development of an accessory opercular ring and the ragged form of the outer acrosome zone. However, too literal an acceptance of the precise sequence of changes should be avoided as it was found in the previous analysis [21] that *Potamonautes* (Fig. 4D), *Calocarcinus* (Fig. 4F) and the two xanthids included were unified by a peripercular rim, remaining well developed in *Potamonautes* (Fig. 4D) and *Calocarcinus* (Fig. 4F), becoming weak in the xanthid *Etisus* (excluded from the present analyses), and lost in *Pilodius* (Fig. 4E) but that this character is ambiguous. When the character was treated as ordered, it was unambiguous, being represented weakly in the ancestor of this clade and in *Etisus* (excluded from the present study), developing from this state to well developed in *Calocarcinus* and *Potamonautes*, and being lost in *Pilodius* [21]. From intuitive studies, xanthids are united by the presence of a ring around the base of the inner acrosome zone, the xanthid ring [15]. In the present study strong development of a peripercular rim occurred independently in *Potamonautes* relative to *Calocarcinus*. Majids are characterized by development of perforation of the operculum and of a posterior median process independently of that in podotremes. *Portunus pelagicus* shows no apomorphies beyond those of basal heterotremes.

The Thoracotremata (Fig. 5A-D) selected for the cladistic studies (This study and [21]) were found to be monophyletic (Fig. 1A, B) on the basis of two unambiguous characters: loss of the acrosome ray zone and movement of the male pores (following that of the female pores basic to heterotremes) onto the sternum. Development of the characteristic apical button in the perforatorium appears ambiguous owing to its alternative absence or loss in *Macrophthalmus* (Fig. 5B). A more detailed investigation of thoracotremes might resolve the issue of whether the button is basic to thoracotremes. In view of the close relationship generally recognized between *Macrophthalmus* and *Ocypode* (Fig. 5C), it seems likely that the absence in *Macrophthalmus* is due to loss of a basic thoracotreme condition.

Concentric lamellae in the acrosome appear to be a development, not seen in *Uca* (Fig. 5A), basal to the higher thoracotremes, *Mictyris* (Fig. 5D), *Ocypode* (Fig. 5C) and *Macrophthalmus* (Fig. 5B). *Uca* differs from the basic thoracotreme condition only in slight shortening of the acrosome.

An interesting outcome of the cladistic analyses is that the "modified xanthid ring" which has been recognized as a characteristic of some thoracotreme sperm and considered to suggest derivation of thoracotremes from a xanthid stock [18] computes as an entirely independent development not related to the xanthid structure (this study and 21]). This does not completely rule out the possibility of derivation from the xanthid ring, however.

Concluding remarks

The parsimony analyses, whether using only spermatozoal characters or spermatozoal and non-spermatozoal characters, provide a remarkable affirmation of the classification of the Brachyura by GUINOT [4, 5] which differed so markedly from pre-existing and, in some schools, still current classifications. Thus the validity of, and phylogenetic justification for, the groupings Podotremata and Heterotremata (though only in *sensu lato*) and Thoracotremata is affirmed. Podotremes and Heterotremata *sensu lato* are confirmed as sister-taxa. Association of the Raninoidea and Cyclodorippoidea is upheld (as sister-groups), each with convincing and unique synapomorphies, but sperm data considered alone do not support alliance of the Homolidae, though equally clearly defined, with this Raninoidea+Cyclodorippoidea couplet and

Source : MNHN, Paris

therefore do not endorse the grouping Archaeobrachyura. Combined spermatozoal and non-spermatozoal data do, however, support the Archaeobrachyura. There is, nevertheless, molecular evidence [31] that raninoids are more closely related to the heterotreme-thoracotreme assemblage than they are to other podotrematous crabs. Within the Podotremata, the Dromiacea *sensu* GUINOT (Dromiidae, Dynomenidae and Homolodromiidae) is confirmed spermatologically as a monophyletic grouping but the discreteness of the three constituent families is not upheld. *Homolodromia* displays a remarkable mixture of dromiid and dynomenid spermatozoal features while lacking any distinctive apomorphy, and does not appear spermatologically to occupy the basal position in the Dromiacea indicated by GUINOT [5, 10] (the apparent agreement of the combined analysis, in this respect, is due solely to the spermatozoal characters.) The Dynomenidae and Dromiidae are each found to be paraphyletic. An 18S rRNA study [31] also found little support for the Dromiidae as a monophyletic group but, unlike the present study, excluded one dromiid from the Brachyura; the two dromiids included in the molecular analysis never formed a clade. In a bootstrap analysis the dromiid *Hypoconcha arcuata* grouped with a hermit crab while *Dromidia antillensis* formed their sister taxon [31]. Examination of the spermatozoa of *Hypoconcha* would be very desirable. Relationships of *Latreillia* sp., the sole representative in the present study of the Latreilliidae and considered an homoloid by GUINOT [5] and GUINOT & RICHER DE FORGES [12], are equivocal. It forms a polytomy either with Homolidae+Raninoidea-Cyclodorippoidea with the combined data set or with Homolidae+Dromiidae-Dynomenidae-Homolodromiidae, for sperm only. The association by GUINOT [5] of the Dorippoidea, Portunoidea, Xanthoidea, and Majoidea in the non-thoracotreme Heterotremata is fully supported spermatologically (calappoids, corystoids, parthenopoids, bellioids and leucosioids, also included by GUINOT, were not included in computations). The Thoracotremata is unequivocally supported as a monophyletic group.

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Source : MNHN, Paris

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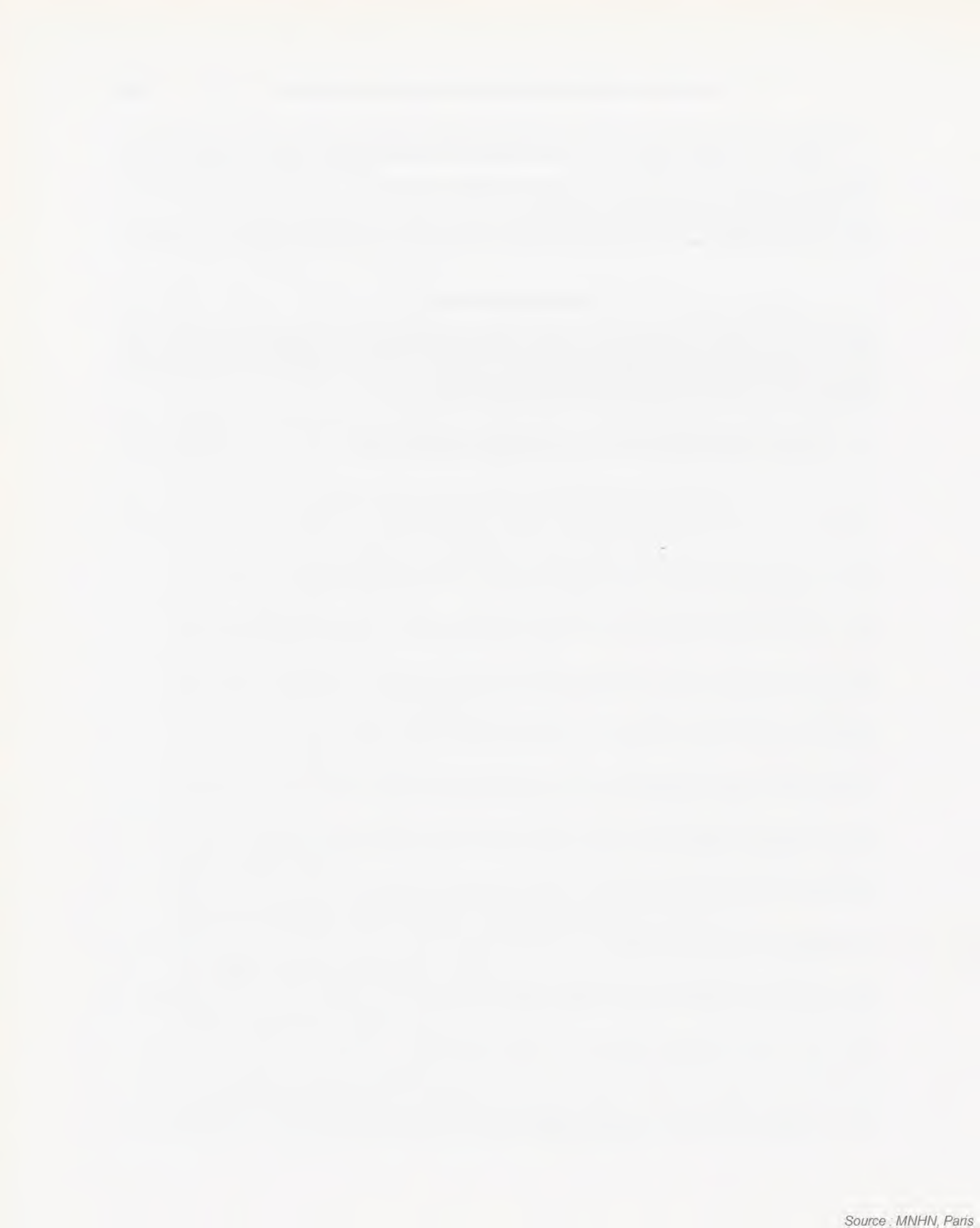
NOTE ADDED IN PROOF

In a valuable paper received as this chapter was going to press, SCHOLTZ & RICHTER (1995) conclude, from a preliminary, mainly morphological analysis, that the Homolodromiidae are the sister-group of all other brachyurans. If this is so, the similarity of the sperm of *Homolodromia* to that of *Paradynomene* is problematical.

SCHOLTZ, G. & RICHTER, S., 1995. — Phylogenetic systematics of the reptantian Decapoda (Crustacea, Malacostraca). *Zoological Journal of the Linnean Society*, **113**: 289-328.

Source : MNHN, Paris



Source : MNHN, Paris

Nuclear Alterations during Spermiogenesis of *Triatoma infestans* (Hemiptera, Reduviidae)

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ABSTRACT

The pattern of spermiogenesis in insects as described for some orders has been generally accepted as the process typical for this taxon. However some species depart from this pattern at specific steps and these differences may be used as taxonomic characteristics. In *Triatoma infestans*, the most striking difference is the lack of nuclear grooves and adjacent membranes which are substituted by coated nuclear protrusions occurring during nuclear elongation. Ten stages of spermiogenesis are recognized and described.

RÉSUMÉ

Altérations nucléaires pendant la spermiogenèse de *Triatoma infestans* (Hemiptera, Reduviidae)

Le processus de spermiogenèse des insectes, tel qu'il a été décrit pour certains ordres, a été généralement accepté comme le processus typique pour ce taxon. Toutefois, certaines espèces se démarquent de ce processus dans des étapes spécifiques et ces différences peuvent être utilisées comme des caractéristiques taxonomiques. Chez *Triatoma infestans*, la différence la plus marquante est l'absence de gouttières nucléaires et de membranes adjacentes qui sont remplacées par des protubérances nucléaires revêtues apparaissant pendant l'élongation nucléaire. Dix stades de spermiogenèse sont reconnus et décrits.

The differentiation of spermatids into spermatozoa is a profound transformation in which all organelles are greatly modified in structure and function, or eliminated when the cell has no further necessity for their contribution towards maturation. To define this process in an orderly manner, it was divided into specific stages by various authors. First described [15] in *Drosophila melanogaster*, the same steps were found in other dipterans such as *Ceratitis capitata* [13] and *Chrysomya megacephala* [11]. Only small differences were noted in other orders such as Orthoptera [16], and Coleoptera [10].

Some isolated aspects of hemipteran spermiogenesis were included in review articles [2, 3]. Aspects of this differentiation process have been thoroughly investigated, such as the formation of the Nebenkern or mitochondrial complex [12]. The nature of the mitochondrial derivatives filled with a paracrystalline structure was investigated in various insects including Hemiptera [4, 14].

DOLDER, H., 1995. — Nuclear alterations during spermiogenesis of *Triatoma infestans* (Hemiptera, Reduviidae). *In*: JAMIESON, B. G. M., AUSIO, J., & JUSTINE, J.-L. (eds), Advances in Spermatozoal Phylogeny and Taxonomy. *Mém. Mus. natn. Hist. nat.*, **166** : 285-289. Paris ISBN : 2-85653-225-X.

Source : MNHN, Paris

The flagellar characteristics of various hemipteran spermatozoa have also been investigated [1, 6-8]. These studies show a strong resemblance between the flagella of the spermatozoa of various genera [7] and even of different families [1].

This is not the case in nuclear and acrosome development. A completely distinct nuclear and acrosome organization was described for the spermatozoa of *Saldula saltatoria* and *Chartoscirta cineta*, where the acrosome is sucker-shaped and the nucleus extends into the flagellum [1]. The development of *Cerris puludum* is another variation in hemipteran spermiogenesis in which complex acrosome and nuclear forms were found in late spermatids [19]. Also in the giant spermatozoon of *Notonecta glauca*, the development of its very long acrosome and nucleus is very different from the pattern known for other Heteroptera [18]. A report on the last stages of spermiogenesis of *Leptocoris trivittatus* (Hemiptera, Corizidae) is more similar to the hemipteran investigated in this study, although acrosome location in a nuclear canal is also distinct from other known species [9]. The most complete description of the nuclear region of an hemipteran during spermiogenesis can be found in relation to *Carabus catenulatus* and *Nepa rubra* [17]. Unfortunately the difference in the methodology used makes some structures difficult to compare.

MATERIAL AND METHODS

Final (5th) stage male nymphs of *Triatoma infestans* were prepared according to routine methods of electron microscopy for determining ultrastructure, using glutaraldehyde and osmium tetroxide. In the case of the specimens seen in figures 2 and 7, the post-fixation in osmium tetroxide was omitted.

RESULTS

The early stages of spermatid development have spherical nuclei, similar to somatic cell nuclei, with finely granular chromatin which condenses only in contact with the nuclear membrane. The subdivision into stages is therefore based on cytoplasmic features, such as the Nebenkern formation in stage 2 (Fig. 1), its separation into two mitochondrial derivatives on each side of the axoneme (stage 3) and their elongation together with the axoneme (stage 4).

The last spherical nucleus stage (5th) is marked by the fusion of the proacrosome vesicle onto the nuclear membrane and its transport to the anterior region of the nucleus. Up to this stage, nucleoli can still be seen, suggesting an active cellular metabolism, necessary for the intense cytoplasmic modifications occurring at this time. Nuclear pores are limited to the region surrounding the basal plaque in which the centriole is anchored (Fig. 2). However, nuclear modification has already begun, as indicated by the first condensations of chromatin into thicker strands (Fig. 3).

In cross sections, the elongating nucleus of stage 6 has two pronounced projections covered by layered dense material (Fig. 4). Chromatin is more densely packed in these

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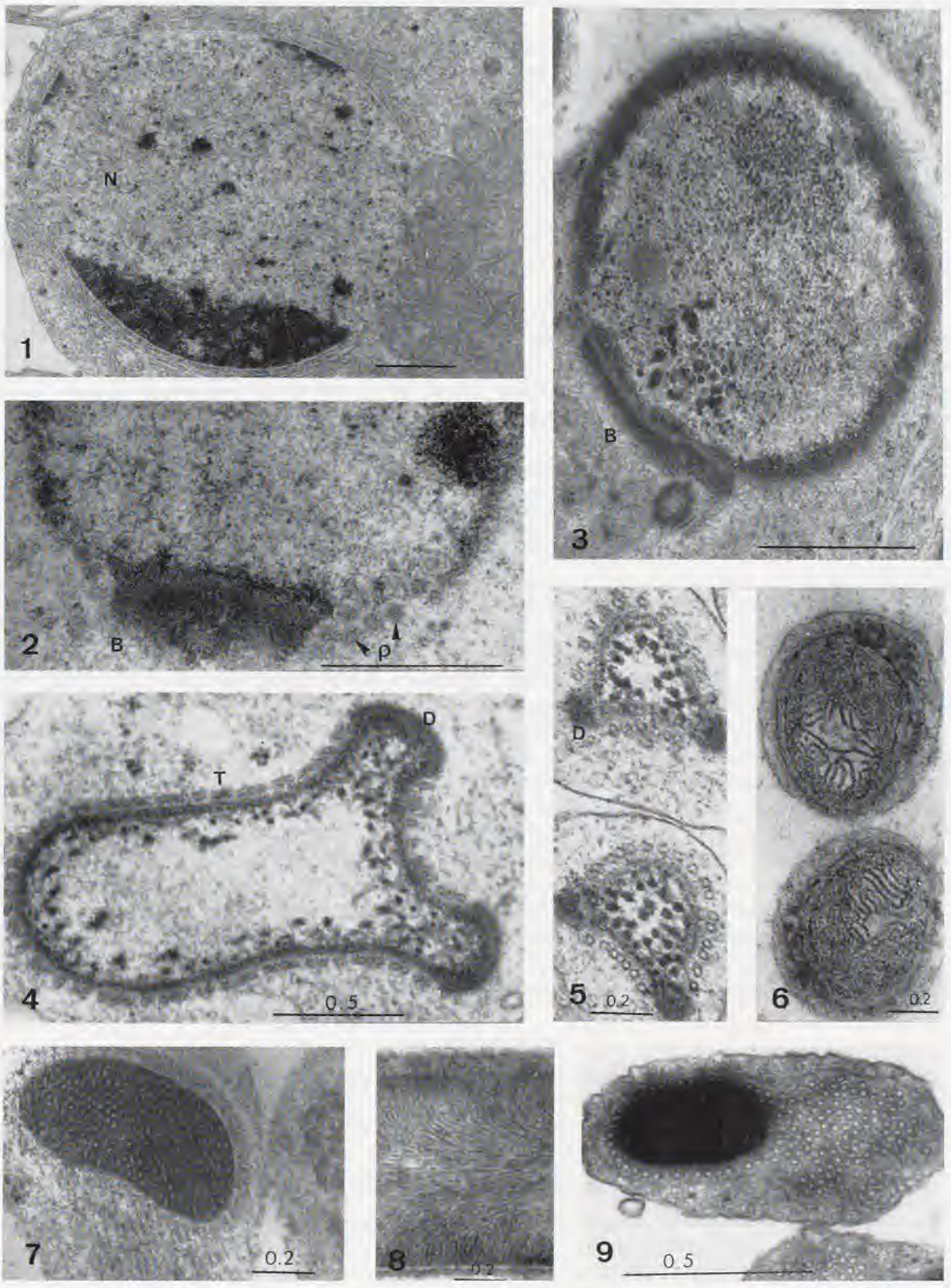
FIGS. 1-9 — Spermiogenesis in *Triatoma infestans*. **1:** Stage 2 spermatid has a round nucleus (N) in which the chromatin begins to condense near the nuclear membrane. A large mitochondrial complex or "Nebenkern" forms at the nuclear base. Bar = 1 µm. **2:** In stage 5, nuclear pores (P) are limited to the periphery of the basal plate (B). **3:** A stage 5 nucleus begins to form chromatin cords of various thicknesses. The basal plate (B) of dense granular material marks the centriolar insertion. **4:** Nuclear elongation and formation of thick chromatic cords identify stage 6. The transversely sectioned nucleus is surrounded by parallel microtubules (T) with the exception of two nuclear projections coated with a dense layer (D). **5:** In stage 7, elimination of the nuclear matrix diminishes the nuclear diameter and the nuclear projections. The dense coating is now limited to small tufts. **6:** Fusion of cords into chromatin lamellae marks stage 8. **7:** A rearrangement of the lamellae to form a regular network indicates stage 9. **8:** A longitudinal section in stage 9 also shows the regular, parallel, chromatin arrangement. **9:** In the last stage (stage 10) of spermatid development, the nucleus is very dense and no substructure of the chromatin can be seen. Cytoplasmic microtubules will be discarded with the excess cytoplasm in the spermatozoon.

Source : MNHN, Paris



Source : MNHN, Paris

protrusions than along the rest of the nuclear membrane which is enclosed externally by a row of parallel microtubules.

In stage 7, elongation is completed and the chromatin has been completely condensed into thick cords (Fig. 5). The nuclear projections are progressively reduced and the dense material which covered them is disorganized into tufts and disintegrates in the cytoplasm.

Stage 8 is identified by lamellar organization of the chromatin (Fig. 6), which rapidly branches out into a close network which identifies stage 9 (Figs. 7 and 8). A longitudinal section shows the compact parallel arrangement of the chromatin lamellae (Fig. 8).

Continued condensation results in a very dense nucleus in which the chromatin organization can no longer be visualized (Fig. 9). Only the sloughing off of the extra cytoplasm and microtubules separate this stage 10 spermatid from the mature spermatozoal structure.

DISCUSSION

Despite the many studies on hemipteran spermatogenesis, these generally concentrate on an interesting aspect of their development and have not detailed the whole process. However, the step by step examination of this process shows that the same subdivision into stages can be applied to this group as has been described for a dipteran [15], an orthopteran [16] and a coleopteran [10].

The active nucleus is practically unmodified, accompanied by major reorganization of the cytoplasmic organelles, during the first stages of spermiogenesis. As nuclear elongation and chromatin condensation begins, these organelles are formed and are gradually modified as maturation proceeds. The next subdivisions of this process are, therefore, based on the nuclear alterations.

Only in stages six and seven are there pronounced differences in relation to the other insects studied. In these previous studies the nucleus forms deep nuclear grooves which are externally lined by the "adjacent membranes", a dense layer of the same thickness as a membrane, which later separates from the nucleus and curls in the cytoplasm where it remains until it is discarded together with the excess cytoplasm and microtubules.

In *Triatoma infestans*, the nuclear protrusions can be described as a new feature, apparently homologous in function with the nuclear grooves. The dense covering of these protrusions is very different from the adjacent membranes, not only in structure but also in its progressive disappearance at the end of stage 7 when the nuclear protrusions are absorbed and the dense material breaks up, becoming indistinguishable in the cytoplasm.

The greater condensation of the chromatin in the protrusions seems to indicate a role in chromatin condensation into thick cords and their organization along the length of the nucleus.

Stages 8 and 9 were rarely encountered and this has been interpreted as an indication that the events of chromatin lamination, network formation and condensation into a very dense, uniform nucleus occur very rapidly. Considering this fact, it may be more appropriate to unite the eighth and ninth stages as a single, more frequently encountered phase of spermiogenesis.

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Source : MNHN, Paris

Source : MNHN, Paris

Sperm Ultrastructure of *Xenos vesparum* (Rossi) and its Significance in the Taxonomy and Phylogeny of Strepsiptera (Insecta)

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ABSTRACT

Sperm ultrastructure of the strepsipteran *Xenos vesparum* was studied by scanning and transmission electron microscopy. The data obtained were compared with those of other strepsipteran species, namely *Xenos moutoni*, *Elenchus tenuicornis*, *E. japonicus* and *Halictophagus chilensis*. From the spermatological point of view, Strepsiptera appear to form a uniform group in which the mature sperm consist of: an elongated head with a monolayered acrosome and a nucleus characterized by an eccentric portion of uncondensed chromatin; and a long tail consisting of a 9+9+2 axoneme flanked by two mitochondrial derivatives of equal size. The spermatozoon of *X. vesparum*, however, shows some species-specific characters such as a longer nucleus which is entirely occupied by condensed chromatin, and two U-shaped mitochondrial derivatives which are devoid of paracrystalline material. These results confirm the sperm morphology as valuable taxonomic character, even if it is not yet sufficient to clarify the controversial phylogenetic affinities of Strepsiptera.

RÉSUMÉ

Ultrastructure du spermatozoïde de *Xenos vesparum* (Rossi) et sa signification pour la taxonomie et la phylogénie des Strepsiptères (Insecta).

L'ultrastructure du spermatozoïde du Strepsiptère *Xenos vesparum* a été étudiée en microscopie électronique à transmission et à balayage. Les données obtenues ont été comparées avec celles des autres Strepsiptères *Xenos moutoni*, *Elenchus tenuicornis*, *E. japonicus* et *Halictophagus chilensis*. D'un point de vue spermatologique, les Strepsiptères semblent former un groupe homogène dans lequel le spermatozoïde mûr consiste en une tête allongée comprenant un acrosome à une seule couche et un noyau caractérisé par une portion excentrique de chromatine non condensée, et une longue queue formée d'un axonème 9+9+2 flanqué de deux dérivés mitochondriaux de taille égale. Toutefois, le spermatozoïde de *X. vesparum* montre quelques caractères spécifiques tels qu'un noyau plus long qui est entièrement occupé par la chromatine condensée, et deux dérivés mitochondriaux en forme de U qui sont dépourvus de matériel paracristallin. Ces résultats confirment la valeur de la morphologie des spermatozoïdes comme caractère taxonomique, même s'ils ne sont pas actuellement suffisant pour éclaircir la position phylogénétique controversée des Strepsiptères.

CARCUPINO, M., PROFILI, G., KATHIRITHAMBY, J., & MAZZINI, M., 1995. — Sperm ultrastructure of *Xenos vesparum* (Rossi) and its significance in the taxonomy and phylogeny of Strepsiptera (Insecta). *In*: JAMIESON, B. G. M., AUSIO, J., & JUSTINE, J.-L. (eds), Advances in Spermatozoal Phylogeny and Taxonomy. *Mém. Mus. nat. Hist. nat.*, **166**: 291-296. Paris ISBN: 2-85653-225-X.

Source : MNHN, Paris

Strepsiptera constitute a small cosmopolitan order of entomophagous parasitic insects which are characterized by extreme sexual dimorphism with free-living, short-lived males and permanently endoparasitic neotenic females (except in the family Mengenillidae).

The ordinal status of the Strepsiptera is still debated. Even up to the present day, several workers, mainly coleopterists, place Strepsiptera as a family (Stylopidae) in the order Coleoptera (beetles) [2, 9, 10, 20] or as a sister group of the Coleoptera [13]. Using classical morphological characters, KINZELBACH [17] reported the plesiomorphies and apomorphies of Strepsiptera. A large number of the apomorphic characters are adaptations to an endoparasitic life; the only apomorphic character uniting Strepsiptera and Coleoptera was found to be the use of the hind wings as the main organ for flight, with consequent specialization of the metathorax for this function.

Recently, KRISTENSEN [18] divided the holometabolic insects into two groups, one consisting of Megaloptera, Raphidioptera, Neuroptera and Coleoptera, the other of Panorpoidea and Hymenoptera, whereas the position of Strepsiptera remained unsolved.

More recently, on the basis of morphological and genetic analysis, a close relationships between Strepsiptera and Diptera has been suggested [13, 21].

Examination of other characters, such as the morphology of the sperm, could provide useful contributions to the phylogeny of Strepsiptera. JAMIESON [11] has coined the term spermiocladistic for the use of sperm ultrastructure in the reconstruction of the phylogeny. There are many examples of spermatological characters (such as the organization of the acrosomal complex and the tail) useful for phylogenetic and evolutionary reconstructions in many animal groups, particularly in insects [for a review see 3, 4, 11].

Descriptions of sperm ultrastructure and spermiogenesis of Strepsiptera have been reported. BACCETTI [5] was the first to describe the ultrastructure of the sperm tail of *Xenos vesparum* Rossi (Stylopidae). Later [8, 15, 16, 19], the sperm organization was studied in four species, namely *Xenos moutoni* De Buysson (Stylopidae), *Elenchus tenuicornis* Kirby, *E. japonicus* Esaki & Hashimoto (Elenchidae) and *Halictophagus chilensis* Hofmann (Halictophagidae). More recently, CARCUPINO *et al.* [9] have examined spermiogenesis in *E. tenuicornis*, while AFZELIUS & DALLAI [1], using a new fixation technique, studied the tail organization in *Stylops* sp.

The present paper aims to provide additional information on the mature sperm and the male reproductive system in *X. vesparum*, and to compare this with all the previous data on the sperm structure in Strepsiptera, in order to contribute to a better understanding of the phylogenetic position of this peculiar Insect order.

MATERIALS AND METHODS

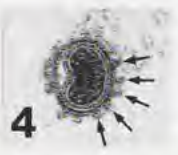
Stylopized wasps *Polistes dominilus* (Christ) were collected in the vicinity of Tübingen (Germany) and brought back to the laboratory. The internal reproductive systems were dissected from free-living males of *X. vesparum* and processed for scanning (SEM) and transmission (TEM) electron microscopy.

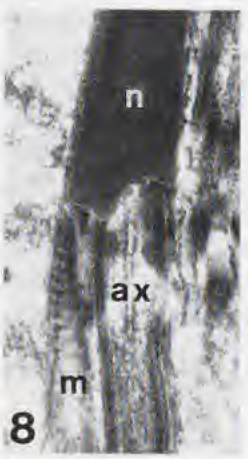
Scanning electron microscopy. Specimens were fixed in Karnovsky's fixative [12] for 2 h at 4° C, rinsed overnight in 0.1 M cacodylate buffer at pH 7.2, post-fixed in similarly buffered 1% osmium tetroxide, dehydrated in a graded ethanol series, dried with the critical point method, gold coated and observed with a JEOL JSM 5200 electron microscope.

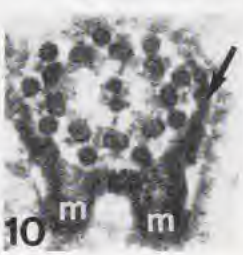
FIG. 1. — SEM micrograph of the internal reproductive system of adult male *Xenos vesparum* showing aedeagus (ae), ejaculatory duct (ed), sperm pump (sp), seminal vesicles (sv), and testes (t). x 215

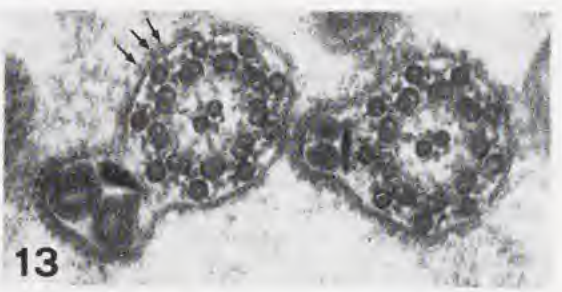
FIGS 2-15. — Longitudinal (2, 8) and cross-sections (3-7, 9-15) at different levels of the spermatozoon of *X. vesparum*. Observe the brush-like glycocalyx at each level of the sperm surface (arrows). **2:** Longitudinal section of the sperm head. x 14 400. **3:** Cross-section of the acrosome. x 90 000. **4-7:** Cross-sections at different level of the kidney-shaped nucleus, from the apex (4) to the base (7). x 90 000. **8-10:** Longitudinal (8) and cross-section (9,10) of the neck region of the sperm tail showing the centriolar area with mitochondrial derivatives (m) characterized by crescent-like appendages (arrows) encircling the axoneme (ax). 8, x 54 000, 9-10, x 90 000. **11-15:** Cross-sections at different levels of the tail with mitochondrial derivatives (m) reducing in size and axoneme (ax) progressively disorganized. x 90 000.

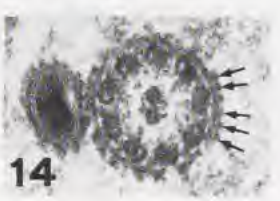
Source : MNHN, Paris

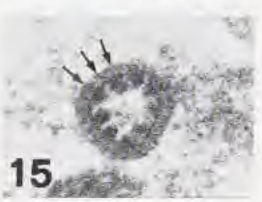












Source : MNHN, Paris

Transmission electron microscopy. Specimens were fixed and dehydrated as above, then embedded in Epon-Araldite mixture. Thin sections were cut in a Reichert Ultracut and a LKB Nova ultramicrotomes, stained with uranyl acetate and lead citrate and observed with a JEOL JEM 1200 EX II electron microscope.

RESULTS

The reproductive system of *X. vesparum* (Fig. 1) includes a pair of elongated testes which open into two large pear-shaped seminal vesicles. The seminal vesicles in turn open separately into a single large muscular sperm pump via two thick vasa deferentia. A single ejaculatory duct emerges from the posterior portion of the sperm pump and terminates in the aedeagus (Fig. 1).

The morphology of the mature sperm is similar to that of the other species of *Xenos* previously examined [19]. The sperm is filiform with an elongated head and a long tail in which three different regions, namely neck region, principal piece and end piece, can be recognized (Figs 2-15).

The head, about 8.5 µm long, is occupied by a monolayered acrosome and a nucleus (Figs 2, 3). In cross-section, the nucleus, which is narrower in the apical portion, is kidney-shaped and appears entirely occupied by compact and electron-dense chromatin (Figs 4-7).

The tail has a simple organization with the axoneme flanked by two long mitochondrial derivatives (Figs 8-13). The axoneme has the common 9+9+2 pattern in which nine accessory tubules, nine microtubular doublets with two dynein arms, and two central microtubules can be recognized (Figs 11-13). The mitochondrial derivatives have typical cristae looking like a series of lamellae which are regularly spaced and aligned at right-angles to one side of the major axis of the derivatives (Fig. 8). No paracrystalline structure seems to be present in the mitochondrial matrix.

The neck region shows a bilocular head-tail junction in which the centriolar region with the beginning of the axoneme and the mitochondrial derivatives is located (Fig. 8). Cross-sections of this region show that the mitochondrial derivatives have a U-shaped appearance with a crescent-shaped appendage encircling the axoneme on each side (Figs 9, 10). These appendages progressively reduce in size until they disappear. Along the principal piece, the axoneme is always flanked by two U-shaped mitochondrial derivatives from which it is separated by a conspicuous membranous sheath (Figs 11, 12). Proceeding toward the endpiece, the mitochondrial derivatives reduce in size and the axoneme becomes disorganized (Figs 13-15). Along the endpiece, axonemal disorganization occurs with the disappearance of the nine accessory tubules first (Fig. 14), then of the two central microtubules, and later of the nine peripheral doublets (Fig. 15).

The entire surface of the sperm, from the acrosome to the endpiece, is covered by a thin brush-like glycocalyx (Figs 3-7, 9-15).

DISCUSSION

The spermatozoon of *Xenos vesparum* is similar to that of other species of Strepsiptera examined previously. Except for a few peculiarities observed in the sperm ultrastructure of each species examined [8, 15, 16, 19], such as different shape of the acrosome, nucleus and mitochondrial derivatives, Strepsiptera were considered a uniform group. The mature sperm is filiform and consists of a monolayered acrosome at the tip of an elongated nucleus which has a portion of uncondensed chromatin in an eccentric position, and a bilocular head-tail junction followed by a 9+9+2 axoneme flanked by two mitochondrial derivatives of equal size.

The mature sperm of *X. vesparum* shows some characters similar to that of *X. moutoni* [19], such as U-shaped mitochondrial derivatives and sperm surface covered by a brush-like glycocalyx, which could be considered as generic characteristics. However, the spermatozoon of *X. vesparum* also shows several species-specific characters. The sperm nucleus is about twice as long as that of the other species examined and lacks the internal portion of uncondensed chromatin. The mitochondrial derivatives have an unusual appearance. Like the typical mitochondrial derivatives, they have cristae with the appearance of a series of lamellae regularly

Source : MNHN, Paris

spaced and aligned orthogonally to the major axis of the sperm, but they lack the paracrystalline material. Mitochondria without any sign of crystallization were also reported in *Stylops* sp. [1].

These data confirm that the morphology of the spermatozoon has real value in providing taxonomic characters, and that the spermatozoon of Strepsiptera does not resemble that of Coleoptera. Coleoptera sperm resemble those of Strepsiptera in having the tail with a 9+9+2 axoneme flanked by two mitochondrial derivatives but differ in having a three layered acrosomal complex (the acrosomal vesicle is located between the periacrosomal cap and the subacrosomal material) and two accessory bodies (for a review see [6, 11]). However, the phylogenetic affinities of Strepsiptera remain unsolved.

Recently, AFZELIUS & DALLAI [1], studying insect sperm tails fixed with a glutaraldehyde-tannic acid mixture, reported that Strepsiptera sperm deviate in several important respects from those of other components of the neuropteroid superorder (Megaloptera, Raphidioptera, Neuroptera and Coleoptera). In particular, the incomplete kidney-shaped accessory axonemal tubules, the lack of the intratubular material and of the accessory bodies distinguished the strepsipteran spermatozoa from those of any other insect groups [1]. The sperm tail of *X. vesparum*, however, as well as those of the other strepsipteran species previously examined [5, 8, 15, 16, 19] shows normal circular accessory tubules. It is not easy to discriminate if this difference is related to specific characteristics or to the new fixative, or to different stages of sperm maturation. In fact, by using the glutaraldehyde-tannic acid mixture, the axonemal structures are better resolved, and therefore the kidney shape might represent the real shape of the strepsipteran accessory tubules. However, the kidney-shaped accessory tubules showed in *Stylops* sp. [1] belong to an immature sperm as demonstrated by the presence of microtubules flanking the mitochondria (see micrograph number 8 in [1]). On this basis, it could be hypothesized that the strepsipteran accessory tubules are kidney-shaped during the differentiation stages and become circular in cross-section at the end of maturation. A survey of mature spermatozoon of *Stylops* sp. could clarify this matter.

Whether these unusual features of the strepsipteran sperm reflect an adaptation to an endoparasitic life, or an isolated systematic position (or both) is still unclear. In fact, related to the endoparasitic life are a very simple female reproductive system [14] and a peculiar mode of insemination and fertilization to which the organization of the sperm could be also related. Further light might be thrown on this problem by a study of sperm morphology in the Mengenillidae, a primitive strepsipteran family in which both males and females are free-living.

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Source : MNHN, Paris

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Source : MNHN, Paris

Characteristics of the spermatozoon of *Cosmopolites sordidus* (Coleoptera: Curculionidae)

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ABSTRACT

The ultrastructure of the spermatozoon of *Cosmopolites sordidus* is similar to that described for other beetles. The acrosome consists of three structures: the perforatorium, the acrosomal vesicle and an extra-acrosomal layer. It is embedded in the nucleus which contains dense, homogeneous chromatin. The flagellum has the typical 9+9+2 microtubule arrangement, two mitochondrial derivatives and two accessory bodies. However, the implantation of these organelles is not in agreement with the description given for the subfamily Rhynchophorinae to which this species belongs. A revision of these characters is needed.

RÉSUMÉ

Caractéristiques du spermatozoïde de *Cosmopolites sordidus* (Coleoptera: Curculionidae)

L'ultrastructure du spermatozoïde de *Cosmopolites sordidus* est similaire à celle décrite pour les autres Coléoptères. L'acrosome est composé de trois structures: le perforatorium, la vésicule acrosomienne et une couche extra-acrosomienne. Il est enchâssé dans le noyau qui contient une chromatine dense et homogène. Le flagelle a la structure typique 9+9+2, deux dérivés mitochondriaux et deux corps accessoires. Toutefois, l'implantation de ces organites ne correspond pas à la description donnée pour la sous-famille des Rhynchophorinae à laquelle cette espèce appartient. Une révision de ces caractères est nécessaire.

Ultrastructural investigations have furnished valuable contributions in relation to the phylogenetic study of many animal groups, including insects. JAMIESON proposed the term spermiocladistics for the use of spermatozoon ultrastructure for phylogenetic reconstruction [8]. Many publications exist along this line; as examples we can cite those dealing with the Chrysomelidae [2] and the superfamily Curculionoidea [5].

While spermatozoal characteristics have been used to define the phylogenetic position of various coleopterans, the position of the *Cosmopolites sordidus* spermatozoon, with regard to its ultrastructural characteristics, is not clear.

LINO NETO, J., & DOLDER, H., 1995. — Characteristics of the spermatozoon of *Cosmopolites sordidus* (Coleoptera: Curculionidae). *In*: JAMIESON, B. G. M., AUSIO, J., & JUSTINE, J.-L. (eds), Advances in Spermatozoal Phylogeny and Taxonomy. *Mém. Mus. natn. Hist. nat.*, **166**: 297-300. Paris ISBN : 2-85653-225-X.

Source : MNHN, Paris

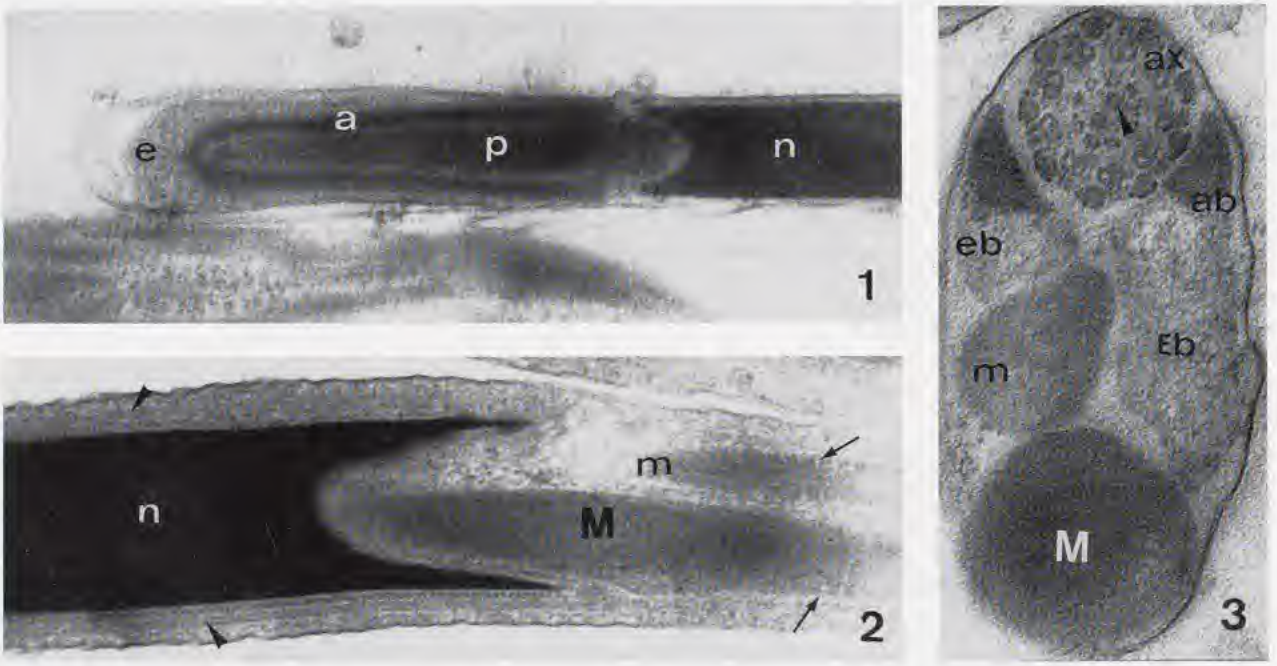
MATERIAL AND METHODS

Adult males were anaesthesized, dissected and their testes fixed by immersion in 3% glutaraldehyde in 0,1 M phosphate buffer at pH 7.2, for 3 hours, post fixed in osmium tetroxide 2% in the same buffer for 1 h, all solutions maintained at 4°C. They were dehydrated with an ethanol series, followed by acetone and embedded in Epon 812. Ultrathin sections were examined with a Zeiss EM 902 electron microscope, after staining with uranyl acetate and lead citrate.

RESULTS

In *Cosmopolites sordidus*, the acrosome measures 1 µm in length and presents three structures: the extra-acrosomal layer, the acrosomal vesicle and the perforatorium. This last structure has its base resting in a concavity of the anterior nuclear surface (Fig. 1).

The nucleus measures 21 µm in length and is totally filled with homogeneous, compact chromatin. The nuclear base forms a cavity which is occupied by the anterior tip of the major mitochondrial derivative (Fig. 2). The flagellum consists of the axoneme, two mitochondrial derivatives and two accessory bodies. In transverse section, the flagellum is oval, with the axoneme and major mitochondrial derivative occupying opposite ends. The axoneme follows the 9+9+2 pattern, with nine accessory microtubules filled with dense material surrounding the nine doublets and two central microtubules, of which, in a perfectly positioned axoneme, the left one is also dense (Fig. 3).



FIGS 1-3 — Spermatozoon of *Cosmopolites sordidus*. **1:** Longitudinal section of the acrosome. The nucleus (n), extra-acrosomal layer (e), acrosomal vesicle (a) and perforatorium (p) can be identified. x 70 000. **2:** Longitudinal section of a late spermatid with the nucleus (N), major mitochondrial derivative (M), minor mitochondrial derivative (m), the expansion of accessory bodies (ab), the major expansion (Eb) and minor expansion (eb) of the accessory bodies. Arrows show the mitochondrial cristae and the arrow heads, the microtubules. x 46 800. **3:** Transverse section of the flagellum, including the axoneme (ax), large mitochondrial derivative (M), minor mitochondrial derivative (m), the major expansion (Eb) and minor expansion (eb) of the accessory bodies (ab). The arrow head indicates the central microtubule not filled with electron dense material. x 82 000.

Source : MNHN, Paris

The major mitochondrial derivative has a diameter equal to twice that of the minor derivative and is almost entirely occupied by material in a paracrystalline arrangement (Fig. 3), although small cristae can still be found (Fig. 2). Cristae are larger in the minor mitochondrial derivative.

The accessory bodies lie parallel to the axoneme, between this structure and the mitochondrial derivatives. Each body has a dense region and a less compact expansion, which is larger for one of the accessory bodies (Fig. 3).

DISCUSSION

The acrosome of *Cosmopolites sordidus* is similar to those of the majority of the Curculionoidea [5, 6] in relation to structure and localization but the subacrosomal lamella was not observed between acrosome and nucleus, as described for other Curculionidae [5]. Crystalline arrangements of acrosomal contents have been found in various insects [4, 13, 15], but no such formation occurs in the acrosome of *C. sordidus*.

The nucleus, as in most Curculionoidea, is long and thin, containing densely compact and homogeneous chromatin. This is contrary to the description for *Stophilus oryzae* L., on which the taxonomic characteristics of the subfamily Rhynchophorinae has been established. This species is claimed not to have a compact nucleus [5].

Mitochondrial derivatives of different sizes have been reported for all known Curculionoidea [6-9, 16]. The internal structures of the two mitochondrial derivatives are similar to those described for *S. multistriatus* [16] and various families of Curculionoidea [5]. The insertion of the major mitochondrial derivative into a cavity of the nucleus is the condition accepted for most Curculionoidea, with the exception of *S. oryzae* [5]. Again, in relation to this species, the mitochondrial derivative was described as being lateral in relation to the nucleus.

The axoneme has the typical arrangement of microtubules described for many insects [1, 3-5, 8, 11, 14]. However, nine accessory microtubules and one of the central microtubules contain dense material in *C. sordidus*, while other insects have dense material in both central microtubules, as well as in the nine peripheral elements [3, 11-13, 15].

The accessory bodies of *C. sordidus* are very similar to these structures in other Curculionoidea previously described [5]. They are not, however, a uniform feature in all coleopterans, often varying in shape, as in the rounded bodies, subdivided into distinct medullar and cortical regions, found in *Divales bipustulatus* [10]. They may even be lacking in *Dermestes frischii* [7].

The expansions of these bodies are less dense and of unequal size in most Curculionidae while in other species they are equal U-shaped as in *S. multistriatus* [9] and *Curculio elephas* [5]. This expansions may also not exist, as in *D. bipustulatus* [10].

The analysis of *C. sordidus* has shown that this species is very similar to the other families of Curculionoidea previously studied [5]. However this species differs from *S. oryzae* which was analysed as a representative of the Rhynchophorinae. In *C. sordidus*, the acrosome is implanted in an anterior depression of the nucleus, while this depression is much shallower or even non-existent in other Curculionidae. Also, there is no subacrosomal lamella in this species, as has been described for other members of the subfamily. The nucleus of *C. sordidus* is densely compacted and has a concavity at its base in which the larger mitochondrial derivative is anchored. This arrangement of the flagellar organelles is also in disagreement with the description of *S. oryzae*, where the mitochondrial derivative was found laterally placed in relation to a nucleus with diffuse chromatin.

In view of these conflicting features, we believe that other species should be investigated so as to confirm the structures which may be adopted as taxonomic characteristics of the Rhynchophororinae.

Source : MNHN, Paris

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Source : MNHN, Paris

Phylogenetic Significance of Axonemal Ultrastructure: Examples from Diptera and Trichoptera

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ABSTRACT

The organization of the axoneme in various insects has been examined by using a tannic acid-containing fixative and been found to differ in a systematic fashion between different insect orders. Within each order, both protofilament number and other axonemal characteristics are relatively constant, however. There are two exceptions to this statement: Diptera and Trichoptera. These two orders were therefore examined in order to see whether the axonemal organization of the various species reflects their systematic position. About 24 dipteran species and 35 trichopteran species were examined in this respect. Axonemal structure in Brachycera is similar to that of Tipulomorpha, which indicates a derivation of Brachycera from that group. Mycetophilidae, rather than Tipulomorpha, seems to be the most primitive dipteran taxon, as judged from the axonemal organization. Axonemes of an examined bibionid species (but not of other members of Bibionomorpha) resemble those of culicomorph species. The axoneme in Trichoptera is characterized by a loss of the outer dynein arms and by the presence of accessory tubules with more than 16 protofilaments. The least modified sperm axoneme is found in the primitive families Rhyacophilidae and Glossosomatidae, which have motile spermatozoa and have 17 and 18 protofilaments, respectively. These families, as other members of Integripalpia (but not Hydroptilidae), have inner dynein arms only and sperm motility, whereas members of Annulipalpia have neither inner nor outer dynein arms and no progressive motility. The variability in axonemal organization is greater in Annulipalpia than in Integripalpia.

RÉSUMÉ

La signification phylogénétique de l'ultrastructure des axonèmes: exemples chez les Diptères et les Trichoptères

L'organisation de l'axonème a été étudiée, grâce à une fixation à l'acide tannique, chez de nombreux insectes et s'est montrée différente parmi les différents ordres d'Insectes, en accord avec la systématique. À l'intérieur de chaque ordre, toutefois, le nombre de protofilaments et les autres caractéristiques de l'axonème sont relativement constants, avec deux exception à cette règle, les Diptères et les Trichoptères. Ces deux ordres ont donc été examinés pour déterminer si l'organisation des axonèmes reflète leur position systématique. Environ 24 espèces de Diptères et 35 espèces de Trichoptères ont été étudiées. La structure de l'axonème chez les Brachycères est similaire à celle des Tipulomorphes, ce qui indique une origine des Brachycères dans ce groupe. D'après l'organisation de l'axonème, les Mycetophilidae, plutôt que les Tipulomorphes, semblent être le taxon de Diptères le plus primitif. Les axonèmes d'une espèce de Bibionidae étudiée (mais pas des autres membres des Bibionomorphes) ressemblent à ceux des Culicomorphes. L'axonème des Trichoptères est caractérisé par la perte des bras de dynéine externes et par la présence de tubules accessoires ayant plus de 16

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Source : MNHN, Paris

protofilaments. L'axonème de spermatozoïde le moins modifié est trouvé dans les familles primitives Rhyacophilidae et Glossosomatidae, qui ont des spermatozoïdes motiles, et respectivement 17 et 18 protofilaments. Ces familles, comme les autres Integripalpia (mais pas les Hydroptilidae), ont seulement des bras de dynéine internes et des spermatozoïdes motiles, alors que les Annulipalpia n'ont ni bras internes ni bras externes de dynéine et n'ont pas de motilité permettant la progression. La variabilité de l'organisation des axonèmes est plus grande chez les Annulipalpia que chez les Integripalpia.

Most insect species have a sperm flagellum that contains a set of microtubules, which surround the nine microtubular doublets and which are named accessory tubules [21]. The axoneme is then said to have a 9+9+2 structure, which is a shorthand notation of 9 accessory tubules, 9 doublets and 2 central microtubules. It was long believed that 9+9+2 axonemes are of uniform appearance within the insect class, but this is far from true.

In a study from 1990 [4] we describe the axoneme of 49 insect species, representing 20 insect orders and show that there is a considerable diversity between different insects. Since that date we have extended the examination to many more species. In fact, axonemes from many species in most orders have been examined by now and this has been done with a new fixation technique that makes it meaningful to study the axoneme at high magnifications. In the resulting micrographs it is possible to count the number of protofilaments in the accessory tubules or in the microtubular doublets and singlets, and it is possible to see a substructure in the lumen of the microtubules or in the so called intertubular material (Intertubular material appears as one or two lumps of electron dense material external to the doublets and between the accessory tubules).

The main findings are summarized in Table 1. It can be concluded that diversity in axonemal structure is related to the systematic position of the animal. Many apomorphic traits can thus be found, such as accessory tubules with 17 protofilaments in the order Phasmida, accessory tubules with 13 protofilaments and no intertubular material in the order Ephemeroptera, and aberrant flagella in the order Thysanoptera. There are also some synapomorphic traits, such as accessory tubules with an elliptic cross-section recorded from the three insect orders Psocoptera, Mallophaga and Anoplura.

Although there is a marked diversity in axonemal structure within the class Insecta, there is a relative invariability within each insect order. There are two exceptions to this statement: Orders Diptera and Trichoptera have families and suborders with markedly different axonemal patterns. The purpose of the present study has been to examine the sperm tail from insects in these two orders in an attempt to see whether any phylogenetic conclusions can be drawn from the ultrastructural data.

MATERIAL AND METHODS

Spermatozoa from the insect species listed in Table 2 have been fixed for one or several days in a fixative that consists of 2 % glutaraldehyde, 1 % tannic acid, and 1.8 % sucrose in a 0.1 M phosphate buffer. This fixative is a modification of the fixative introduced by MIZUHIRA & FUTAESAKU [23]. Post-fixation and block-staining was performed with 1 % uranyl acetate in distilled water. No osmium tetroxide fixation was used. After dehydration in ethanols and embedding in epoxy resins, sections have been made and examined at a direct magnification of 50 000 or higher.

Tannic acid is thought to act as a mordant that encapsulates proteins; uranyl acetate is thought to contrast the tannic acid. Globular protein such as a tubulin in the microtubular wall will become visible as a lucid spot surrounded by an electron dense shell. Fibrous proteins, such as those in the spokes, may however appear as electron dense structures. Interpretation of the electron micrographs is not trivial.

OBSERVATIONS

Diptera

The axoneme of *Orfelia* sp. (Family Mycetophilidae) is of the 9+9+2 type. Its doublets have both inner and outer dynein arms. The accessory tubules have 16 protofilaments and the

Source : MNHN, Paris

intertubular material between two accessory tubules appears as two electron dense masses, i.e. a smaller unit attached to the accessory tubule and a larger one attached to the doublet; occasionally the two masses are confluent and form a single mass. In these respects the axoneme resembles that of examined members of several insect orders, such as Zygentoma, Plecoptera, Embioptera, Orthoptera, Dermaptera, Megaloptera, Raphidioptera, Planipennia, Coleoptera, Lepidoptera, and Hymenoptera. The cross-sectioned flagellum of an *Orfelia* spermatozoon differs from that of the other insects in having a single small mitochondrial derivative and differs also in other respects from those within other insect orders, but the axoneme has a morphology that can be regarded as plesiomorphic for insects.

The axoneme from some other members of the Mycetophilidae family has other patterns: *Tarnania* 2+9+2 and *Exechia seriata* 7+9+2 (both with 16 protofilaments in their accessory tubules) (Fig. 1), *Keroplatus reaumuri* 9+2, and *Boletina* sp. 9+9+2 with accessory tubules consisting of 15 protofilaments (Fig. 2). Such a variety of different axonemal patterns has not been described from any other insect family apart from Cecidomyiidae, in which there are highly aberrant spermatozoa.

The three examined members of family Chironomidae all have an axoneme of the 9+9+2 type and the accessory tubules have 15 protofilaments. The accessory tubules of examined members of the three families Dixidae, Culicidae (Fig. 3), and Bibionidae (Fig. 4) also have 15 protofilaments. However, their axoneme is of the 9+9+1 type as the two central microtubules are replaced by a central rod or cylinder in the three families.

In the examined species of families Trichoceridae and Tipulidae (Fig. 5) the axoneme is of the 9+9+2 type but the accessory tubules have 13 protofilaments. In this respect they resemble all Brachycera species examined so far [15, 24] (Fig. 6). Another characteristic shared by the axonemes in Tipulidae, Trichoceridae and Brachycera is the prominence of the spoke head which usually seems to be double. Within Brachycera the intertubular material contains a straight row of inclusion bodies [15].

Trichoptera

Most specialists on Trichoptera agree that the order can be divided into two subgroups, Integripalpia and Annulipalpia, although some uncertainty remains as to whether families Rhyacophilidae, Glossosomatidae and Hydroptilidae belong to one suborder or the other. These three families are usually regarded as the most primitive ones within the Trichoptera. In the family Rhyacophilidae the axoneme has a 9+9+2 structure, the doublets have an inner dynein arm but no outer one, and the accessory tubules have 17 protofilaments along most of their length (Fig. 7), although 16 close to the posterior end. In the examined member of the family Glossosomatidae the axoneme has the same characteristics, except that there are 18 rather than 17 protofilaments along most of the length of the accessory tubules. Spermatozoa from Rhyacophilidae and Glossosomatidae show progressive motility, whereas spermatozoa from the examined members of the family Hydroptilidae lack a flagellum and are immotile.

The integripalpian family Leptoceridae also has a 9+9+2 axoneme, inner dynein arms only, and accessory tubules with 18 protofilaments (Fig. 8). The axoneme differs from that in Glossosomatidae, however, in that the two central microtubules always occupy an eccentric position. Axonemes in the three Integripalpia families Limnephilidae (Fig. 9), Goeridae and Odontoceridae (Fig. 10), also resemble those in the family Glossosomatidae, although the number of protofilaments is 19 along most of the length, decreasing to 16 distally. The two members of the family Sericostomatidae, finally, have 20 protofilaments proximally, the highest number recorded from any insect or other animal. Posteriorly the number decreases to 16 near the distal end. An accessory body lies lateral to the axoneme.

Source : MNHN, Paris

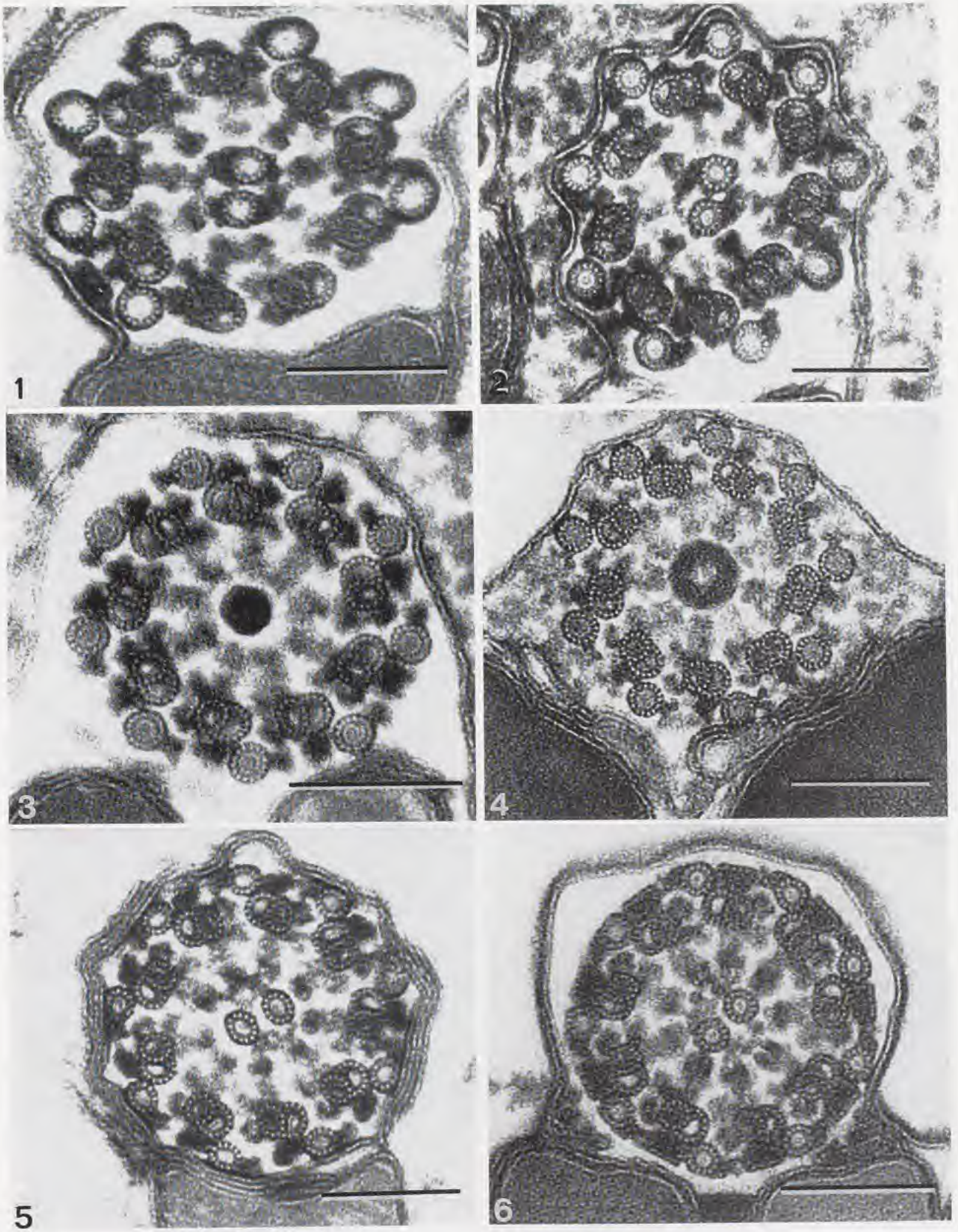
TABLE 1. — Some characteristics of the sperm tail axoneme in examined members of insects from the various insect orders

Abbreviations in the Table are: AT = accessory tubule, IM = intertubular material, OA = outer dynein arms, pfs = protofilaments, the formula 9+9+2 signifies the number of accessory tubules, microtubular doublets, and central microtubules, respectively.

Taxon	Axonemal features	Reference
Protura	no AT, 12+0, 16+0 or otherwise	[19]
Diplura	AT with 13 pfs, ATs arranged in straight line	[6]
Archeognatha	AT with 16 pfs, ATs arranged in two groups	[6]
Zygentoma	AT with 16 pfs	[6]
Odonata	AT with 16 pfs, one IM	[6]
Ephemeroptera	AT with 13 pfs, no IM, no OA, 9+9+0 or 9+9+1	[2, 6]
Plecoptera	AT with 16 pfs, two IMs	[2]
Embioptera	AT with 16 pfs	unpublished
Isoptera	no ATs, usually aflagellate	[6]
Blattodea	AT with 16 pfs, dense lumen in all microtubules	[6]
Mantodea	AT with 16 pfs, IM with 9 inclusion bodies	[6]
Phasmida	AT with 17 pfs, two IMs	[1, 6]
Orthoptera	AT with 16 pfs, prominent IM	[6, 9]
Dermaptera	AT with 16 pfs, prominent IM	[6]
Psocoptera	AT with 13 pfs, compressed ATs, AT lumen dense	[8]
Mallophaga	AT with 13 pfs, compressed ATs, AT lumen dense	[6]
Anoplura	AT with 13 pfs, compressed ATs, AT lumen dense	[6]
Thysanoptera	no AT, 27 microtubules of three kinds	[12]
Hemiptera	AT with 16 pfs, AT and A-tubule lumen dense	[6]
Homoptera	AT with 16 pfs, AT and A-tubule lumen dense	[6]
Megaloptera	AT with 16 pfs, two IMs	[3]
Raphidioptera	AT with 16 pfs, two IMs	[3, 6]
Planipennia	AT with 16 pfs, two IMs	[3]
Coleoptera	AT with 16 pfs, two IMs	[3, 6]
Strepsiptera	AT incomplete and containing an inclusion, no IM	[3, 9]
Mecoptera	no AT	[6]
Hymenoptera	AT with 16 pfs	[6]
Trichoptera	AT with more than 16 pfs, no OA	this paper
Lepidoptera	AT with 16 pfs	[6]
Diptera	AT with 13, 15, or 16 pfs	this paper
Siphonaptera	no AT	[21]

FIGS 1-6. Axonemes from various dipterans: **1:** *Exechia seriata*; **2:** *Boletina* sp.; **3:** *Culex pipiens*; **4:** *Bibio* sp.; **5:** *Tipula* sp.; **6:** *Scatophaga* sp. Scale bar 0.1 µm.

Source : MNHN, Paris



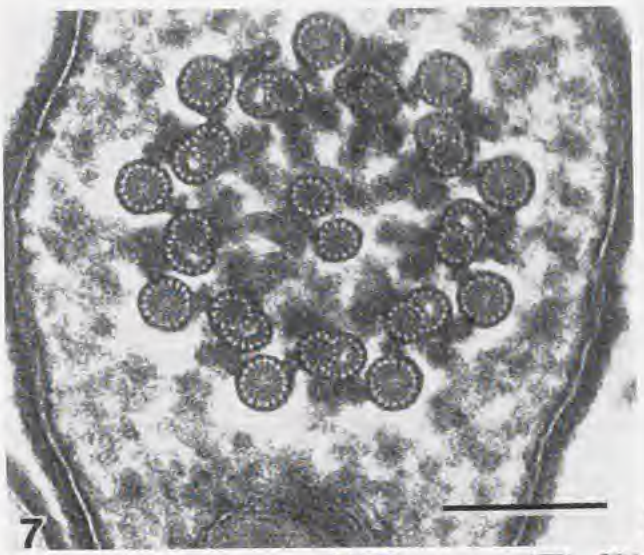
Source : MNHN, Paris

TABLE 2. — Species investigated and sources of data

Group, Species	Family	Reference
DIPTERA NEMATOCERA		
Orfelia sp.	Mycetophilidae	[15]
Exechia seriata (Meigen)	Mycetophilidae	[15]
Boletina sp.	Mycetophilidae	[15]
Keroplatus reaumurii Dufour	Mycetophilidae	[15]
Asphondylia ruebsaameni Kertész	Cecidomyiidae	[18, 22]
Sciara sp.	Sciaridae	[15]
Chironomus (three unidentified species)	Chironomidae	[15]
Culex pipiens L.	Culicidae	[15]
Anopheles maculipennis Meigen	Culicidae	[15]
Dixa sp.	Dixidae	[15]
Bibio sp.	Bibionidae	[2, 15]
Trichocera hiemalis De Geer	Trichoceridae	[15]
Tipula sp.	Tipulidae	[15]
DIPTERA BRACHYCERA		
Bombylius sp.	Bombyliidae	[15]
Ramphomyia sp.	Empididae	[15]
Drosophila melanogaster Meigen	Drosophilidae	[7]
Bactrocera (=*Dacus*) *oleae* (Gmelin)	Tephritidae	[7]
Ceratitis capitata (Wied.)	Tephritidae	[9, 15]
Scatophaga sp.	Scatophagidae	[15]
Calliphora vomitoria L.	Calliphoridae	[15]
Gasterophilus intestinalis De Geer	Gasterophilidae	[15]
TRICHOPTERA INTEGRIPALPIA		
Rhyacophila (Rhyacophila) foliacea Moretti	Rhyacophilidae	[17]
Rhyacophila (Rhyacophila) dorsalis (Curtis)	Rhyacophilidae	[17]
Rhyacophila (Pararhyacophila) italica Moretti	Rhyacophilidae	[17]
Rhyacophila (Hyporhyacophila) tristis Pictet	Rhyacophilidae	[17]
Catagapetus nigrans McLachlan	Glossosomatidae	[17]
Stactobia caspersi Ulmer	Hydroptilidae	[11]
Orthotrichia costalis (Curtis)	Hydroptilidae	[11]
Hydroptila aegyptia Ulmer	Hydroptilidae	[11]
Hydroptila angulata Mosely	Hydroptilidae	[11]
Hydroptila forcipata Eaton	Hydroptilidae	[11]
Hydroptila tineoides Dalman	Hydroptilidae	[11]
Oecetis furva Rambur	Leptoceridae	[10]
Leptocerus tineiformis Curtis	Leptoceridae	[10]
Mystacides azurea L.	Leptoceridae	[10]
Odontocerum albicorne Scopoli	Odontoceridae	[10, 13]
Sericostoma italicum Moretti	Sericostomatidae	[10]
Sericostoma pedemontanum McLachlan	Sericostomatidae	[10]
Silo mediterraneus saturniae Moretti	Goeridae	[10]
Leptodrusus budtzi Ulmer	Limnephilidae	[10]
Limnephilus bipunctatus Curtis	Limnephilidae	[10]
Limnephilus rhombicus (L.)	Limnephilidae	[10]
Glyphotaelius pellucidus Retzius	Limnephilidae	[10]
Potamophylax cingulatus (Stephens)	Limnephilidae	[10]
Melampophylax melampus McLachlan	Limnephilidae	[10]
Stenophylax permistus McLachlan	Limnephilidae	[2, 10]
Micropterna sequax McLachlan	Limnephilidae	[10]
Chaetopteryx gessneri McLachlan	Limnephilidae	[10]
TRICHOPTERA ANNULIPALPIA		
Philopotamus ludificatus McLachlan	Philopotamidae	[16]
Philopotamus montanus Donovan	Philopotamidae	[2, 16]
Wormaldia occipitalis Pictet	Philopotamidae	[16]
Wormaldia copiosa McLachlan	Philopotamidae	[16]
Plectrocnemia geniculata McLachlan	Polycentropodidae	[16]
Polycentropus mortoni Moseley	Polycentropodidae	[16]
Polycentropus irroratus Curtis	Polycentropodidae	[16]
Cyrnus trimaculatus Curtis	Polycentropodidae	[16]
Hydropsyche pellucidula Curtis	Hydropsychidae	[16]

FIGS 7-12. — Axonemes of some trichopterans. **7:** *Rhyacophila foliacea*; **8:** *Leptocerus tineiformis*; **9:** *Potamophylax cingulatus*; **10:** *Odontocerum albicorne*; **11:** *Wormaldia copiosa*; **12:** *Philopotamus montanus*. Scale bar 0.1 µm.

Source : MNHN, Paris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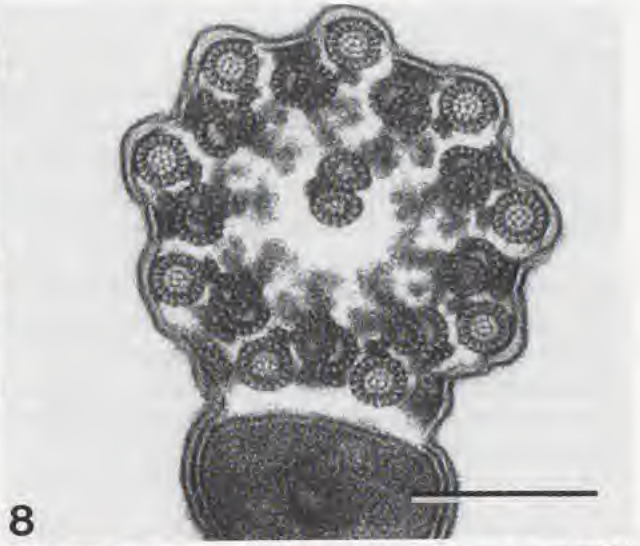
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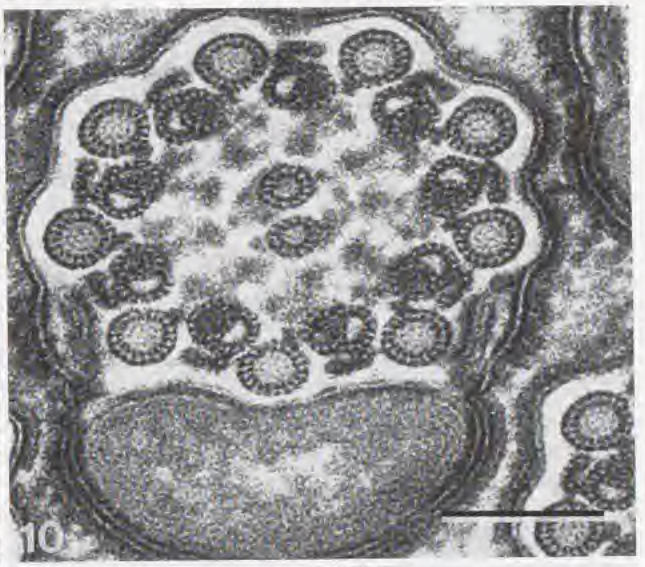
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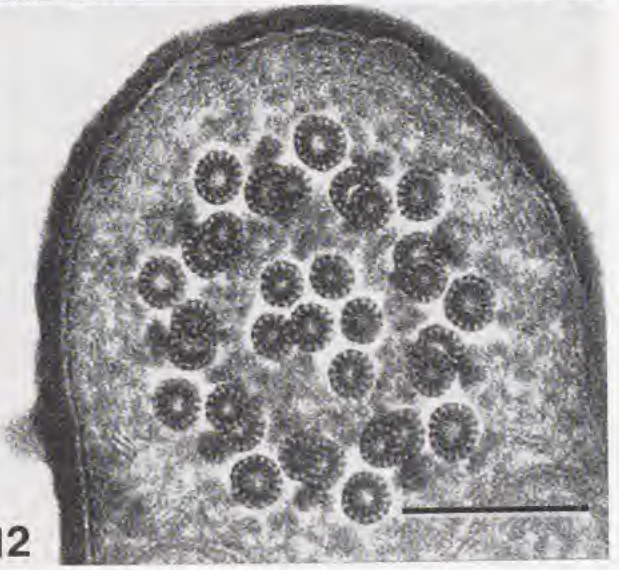
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Source : MNHN, Paris

Members of the suborder Annulipalpia lack inner and outer dynein arms. Hence they have no regular, flagellar movements. Weak vibrations have been observed in *Philopotamus* spermatozoa, when remaining in the testicular fluid, however. The philopotamid species *Wormaldia copiosa* has a unique axonemal pattern, 13+13+v, where v stands for a cylindrical vesicle along the central axis of the axoneme (Fig. 11), whereas another member of the same genus, *W. occipitalis*, has a similar axoneme although with the formula 9+9+v. In examined members of *Philopotamus* the axonemal pattern is 9+9+7 and the seven central microtubules consist of 13 or 14 protofilaments (Fig. 12).

The family Polycentropodidae is related to the Philopotamidae. Its members have lost the accessory tubules and have a 9+7 axoneme, in which the central microtubules have 13 or 14 protofilaments. Also related to these families are the Hydropsychidae, whose members have a highly aberrant sperm morphology. Whereas a section just distal to the centriole shows nine axonemal doublets, sections posterior to that level show a great number of microtubular singlets and doublets, some enclosed in the cell body, others contained in finger-like rodlets projecting from the cell body.

DISCUSSION

Diptera

The suborder Nematocera is generally considered to be more primitive than the Brachycera and its subgroup Tipulomorpha (= Tipulidae + Trichoceridae) and to be the sister group to all other Diptera [5, 20]. Tipulomorpha is thus regarded as the lowest branch of the dipteran phylogenetic tree. Our data on the sperm axoneme is not compatible with this opinion. Rather, it seems to us that the family Mycetophilidae is the most primitive dipteran taxon. Only in this family has the plesiomorphic axonemal pattern - 9+9+2 and accessory tubules with 16 protofilaments - been found. Whether data from molecular biology will confirm that Mycetophilidae is the most primitive extant dipteran group, or whether data will show the Tipulimorpha to be so, is a matter that probably will be settled soon.

As mentioned above, some axonemal characteristics are shared between the examined members of Tipulomorpha and the Brachycera: accessory tubules with 13 protofilaments and spoke heads that appear double. We believe that these are synapomorphies shared by these groups and that Brachycera is derived from the tipulomorph group. According to HENNIG [20] Nematocera contains four large groups: Tipulomorpha, Psychodomorpha, Culicomorpha (containing, amongst other families, Dixidae, Culicidae, Simulidae and Chironomidae), and Bibionomorpha (with Bibionidae, Mycetophilidae, Cecidomyidae, Sciaridae). Members of Psychodoidea have an axoneme of the 9+9+0 type [14], but nothing is known about the number of protofilaments in the accessory tubules.

The examined members of the culicomorph families Dixidae, Culicidae, and Chironomidae share an axonemal character: accessory tubules with 15 protofilaments. This number of protofilaments has been found also in the examined member of Bibionidae and in the mycetophilid *Boletina*, but otherwise is a rare feature. Whereas the chironomids have a 9+9+2 axoneme and the simulid a 9+9+3 one [4], culicid and bibionid flies have a central rod rather than two central microtubules, a 9+9+'1' or sometimes a 9+9+0 pattern. This finding indicates that Bibionidae may be closer related to Culicomorpha than to other families of Bibionomorpha or that Culicomorpha is derived from Bibionomorpha . The sperm tails of members of Cecidomyiidae and Sciaridae, finally, have highly aberrant axonemes.

Trichoptera

Based, *inter alia*, on their free-living larvae, the families Rhyacophilidae, Glossosomatidae, and Hydroptilidae are considered to be the most primitive trichopterans. The loss of a sperm tail in Hydroptilidae prevents any comparative study based on those characters that are treated here. The

Source : MNHN, Paris

relatively few modifications from the plesiomorphic insect axoneme in the other two families is compatible with the opinion that they are primitive.

The division of Trichoptera into two suborders, Integripalpia and Annulipalpia, is based on the morphology of the palps. If only the sperm axoneme were considered, Trichoptera could also be divided into two groups, one possessing inner dynein arms and flagellated spermatozoa, the other without these characteristics. This division would be identical to the classical one and the two families Rhyacophilidae and Glossosomatidae would be classified as belonging to Integripalpia. The inclusion of these families (and of Hydroptilidae) into Integripalpia is also advocated by ROSS [25].

The various species of Integripalpia have axonemal patterns that are characteristic of the families to which they belong. No significant differences have been found between the three families Limnephilidae, Goeridae and Odontoceridae. Within Annulipalpia there may be a greater variability between members of the same family or even the same genus. Since the flagellum is unable to propagate the spermatozoon, any mutation that changes the pattern from a regular 9+9+2 pattern can be regarded as neutral, in that it will not affect sperm functions. A greater diversity of the axonemal pattern can hence be expected and has thus been recorded.

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Source : MNHN, Paris

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Vertebrates

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Comparative Morphology of the Sperm in Chondrichthyan Fishes

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ABSTRACT

The external features of the spermatozoa of 35 species belonging to 19 families in 12 orders were observed with light and scanning electron microscopes. Six characters, i.e., type of sperm aggregate, number of helical gyres, total, head, midpiece and flagellum length, were used as an operational taxonomic unit for the computation of the resemblance of the sperm among species in a cluster analysis. The sperm of Orectolobiformes, Lamniformes, Carcharhiniformes, Torpediniformes and Rajiformes form sperm aggregates: spermatozeugmata and spermatophores. The sperm of 29 species are helical from the tip of the head to the midpiece, but *Chlamydoselachus anguineus*, *Dalatias licha*, *Etmopterus* spp., and *Squatina japonica* do not display a helical form. The sperm length ranges from 93 µm to 224 µm. The sperm of holocephalans have a shorter head and longer flagellum in proportion to that of elasmobranchs. The results of the cluster analysis suggest the external features of the sperm show a similarity within the genus and/or family.

RÉSUMÉ

Morphologie comparée des spermatozoïdes des poissons Chondrichtyens

La morphologie externe des spermatozoïdes de 35 espèces appartenant à 19 familles dans 12 ordres a été observée en microscopie photonique et à balayage. Six caractères, le type d'agrégation des spermatozoïdes, le nombre de tour de l'hélicoïde, la longueur du spermatozoïde, de la tête, de la pièce intermédiaire, du flagelle, ont été utilisés comme unités taxonomiques opérationnelles pour le calcul de la ressemblance des spermatozoïdes parmi les espèces par une méthode agglomérative. Les spermatozoïdes des Orectolobiformes, Lamniformes, Carcharhiniformes, Torpediniformes et Rajiformes forment des agrégations: spermatozeugmata et spermatophores. Les spermatozoïdes de 29 espèces sont hélicoïdaux à partir de l'extrémité de la tête jusqu'à la pièce intermédiaire, mais *Chlamydoselachus anguineus*, *Dalatias licha*, *Etmopterus* spp. et *Squatina japonica* n'ont pas de spermatozoïdes hélicoïdaux. La longueur des spermatozoïdes varie de 93 µm à 224 µm. Les spermatozoïdes des Holocéphales ont une tête plus courte et un flagelle plus long que les Élasmobranches. Les résultats de l'analyse par méthode agglomérative suggèrent que la morphologie externe des spermatozoïdes est similaire à l'intérieur des genres et/ou des familles.

Chondrichthyans are a small group of about 900 species, compared with osteichthyans. They include two subclasses, 14 orders, and 51 families [1, 9]. Recently, the systematics and phylogeny of elasmobranchs have been considered using the external, skeletal and muscular systems of the body, and new schemes have been presented [2, 12]. Female chondrichthyan

TANAKA, S., KUROKAWA, H., & HARA, M., 1995. — Comparative morphology of the sperm in chondrichthyan fishes. *In*: JAMIESON, B. G. M., AUSIO, J., & JUSTINE, J.-L. (eds), Advances in Spermatozoal Phylogeny and Taxonomy. *Mém. Mus. natn. Hist. nat.*, **166** : 313-320. Paris ISBN : 2-85653-225-X.

Source : MNHN, Paris

fishes have diverse and complex reproductive styles, which have also been used as characters for consideration of their systematics and phylogeny [3, 10]. In contrast, the reproduction of male fishes is simple. Claspers, which are copulatory organs, differ in structure among species. Therefore, the claspers have traditionally been used to provide characters in taxonomy and systematics [5, 7].

During the last decade, electron microscopy (EM) has been refined. It provides new information on the fine structure and function of organs, tissues and cells in various animals. The sperm of many fishes has been observed with EM, and the interrelationships of fishes have been discussed in relation to the structure of the spermatozoa [6, 8]. However, comparative studies on the sperm of chondrichthyan fishes are few [4]. The present paper deals with the comparative morphology of the sperm in 35 chondrichthyan fishes on the basis of observations with light and scanning electron microscopes.

MATERIALS AND METHODS

Chondrichthyan fishes, belonging to 12 orders, 19 families and 35 species, were collected on the northeast coast of the United States, and in Suruga Bay and adjacent waters of central Japan from 1990 to 1994 (Table 1). Spermatozoa, seminal fluids and tissues were taken from various portions of the reproductive tract of the specimens just after the death. For observing under light microscopy, they were fixed in 10% neutral formalin. Sperm smears were prepared by smearing and air-drying seminal fluids on glass slides, and were used for measuring the dimensions of 30 sperm under Nomarski differential interference microscopy. For scanning electron microscopy (SEM), they were immersed in a cold fixative containing 2% paraformaldehyde and 2% glutaraldehyde in a 0.1 M cacodylate buffer (pH 7.3) with 10% sucrose added. They were rinsed in the above buffer, post fixed in 2% osmium tetroxide in the same buffer, dehydrated in graded ethanol, and displaced with t-butyl alcohol, frozen and dried in a vacuum desiccator. They were sputter-coated with gold, and observed and photographed using an Akashi ABT-55 scanning electron microscope.

For numerical taxonomy on the sperm of each species, six characters of the sperm: type of sperm aggregate, number of helical gyres, total length, head length, midpiece length, and flagellum length, were used as an operational taxonomic unit (OTU). The following five types of sperm aggregate were recognized: 1. a solitary sperm or sperm clump, 2. single-layered spermatozeugma, 3. compound spermatozeugma, 4. "rice-grain" type of spermatophore, 5. atypical rod shaped spermatophore [11]. The types were scored from 1 to 5, respectively. Data sets containing each character were standardized; values of each character were calculated as units of standard deviation from the mean value of each character. The correlation coefficient as a measure of the overall similarity was computed by comparison of each OTU pair. Clusters of the OTUs were created under the unweighted pair-group method using arithmetic averages (UPGMA).

RESULTS

Observations with light and scanning electron microscopes.

Formation of sperm aggregates. The sperm were separate within secretions in the epididymis of all species. In *Orectolobus japonicus*, 3 species of Lamniformes, 13 species of Carcharhiniformes, *Torpedo tokionis* and *Raja eglanteria*, the sperm in the ductus deferens formed clumps with heads adhering. They formed sperm aggregates in the ampulla ductus deferentis as reported in [11]. The type of sperm aggregate for each species is shown in Table 2. Single layered spermatozeugmata were found in 10 species of Carcharhiniformes except *Prionace glauca* and *Sphyrna lewini*, *T. tokionis*, and *R. eglanteria* (Fig. 1A). *O. japonicus* formed compound spermatozeugmata (Fig. 1B). The sperm aggregates of *P. glauca*, *S. lewini*, *Carcharias taurus*, and *Isurus oxyrinchus* have been described in detail [11]. *Alopias pelagicus* possessed rice-grain typed spermatophores. In *Heterodontus japonicus* and *Squatina japonica*, the sperm gathered in clumps in the ductus deferens as in the above species, and retained this arrangement in the ampulla ductus deferentis. Though *Chlamydoselachus anguineus*, 9 species of Squaliformes, *Rhinobatos schlegelii*, and 2 species of Myliobatiformes made sperm clumps temporarily, most of the sperm were solitary in the ampulla. The sperm of Chimaeriformes also formed clumps in the ductus deferens by adhesion of the midpieces, a condition differing from elasmobranchs. However, the sperm clumps were not found in the seminal fluid from the distal end of the ampulla.

Source : MNHN, Paris

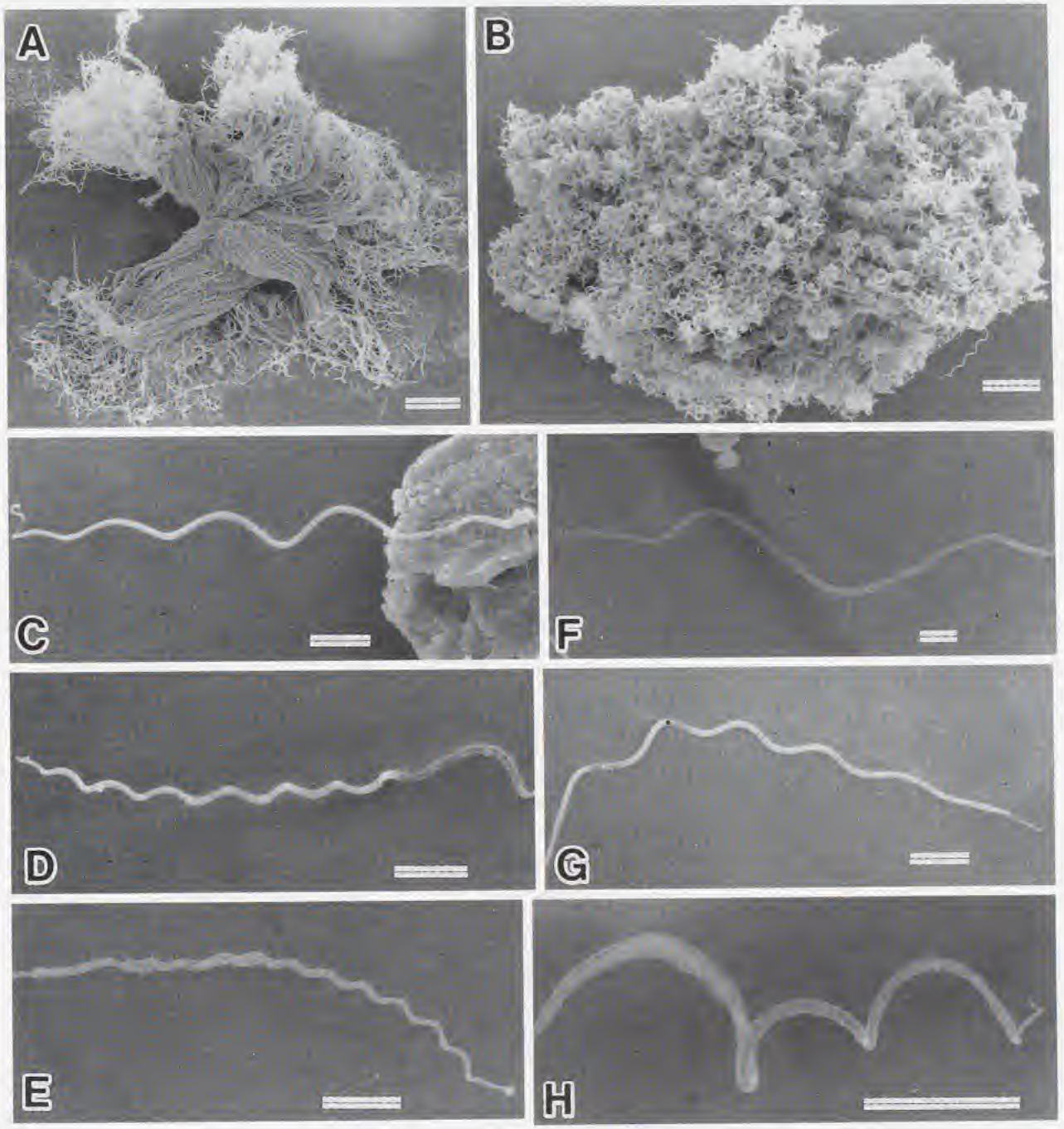

FIG. 1. — Sperm aggregate and the head and midpiece of sperm in several chondrichthyan fishes. **A:** Single layered spermatozeugma of *Galeus nipponensis*. **B:** Compound spermatozeugma of *Orectolobus japonicus*. **C:** Sperm of *Isurus oxyrinchus*. **D:** Sperm of *Mustelus griseus*. **E:** Sperm of *Sphyrna lewini*. **F:** Sperm of *Dalatias licha*. **G:** Sperm of *Rhinobatos schlegelii*. **H:** Sperm of *Chimaera phantasma*. Scale indicates 25 µm in A and B, and 5 µm in C to H.

Source : MNHN, Paris

TABLE 1. — Species and sample size of specimens from which sperm were obtained.

Heterodontiformes	
Heterodontidae	
Heterodontus japonicus	1
Orectolobiformes	
Orectolobidae	
Orectolobus japonicus	2
Lamniformes	
Odontaspididae	
Carcharias taurus	1
Alopiidae	
Alopias pelagicus	1
Lamnidae	
Isurus oxyrinchus	2
Carcharhiniformes	
Scyliorhinidae	
Cephaloscyllium umbratile	2
Galeus eastmani	5
Galeus nipponensis	3
Triakidae	
Hemitriakis japanica	4
Mustelus canis	1
Mustelus griseus	1
Mustelus manazo	2
Carcharhinidae	
Carcharhinus plumbeus	1
Galeocerdo cuvier	1
Prionace glauca	3
Sphyrnidae	
Sphyrna lewini	3
Hexanchiformes	
Chlamydoselachidae	
Chlamydoselachus anguineus	1
Squaliformes	
Squalidae	
Centroscymnus owstoni	3
Dalatias licha	2
Deania calcea	2
Deania historicosa	5
Etmopterus brachyurus	3
Etmopterus molleri	1
Etmopterus pusillus	2
Squalus brevirostris	2
Squalus japonicus	3
Squatiniformes	
Squatinidae	
Squatina japonica	1
Rhinobatiformes	
Rhinobatidae	
Rhinobatos schlegelii	1
Torpediniformes	
Torpedinidae	
Torpedo tokionis	2
Rajiformes	
Rajidae	
Raja eglanteria	2
Myliobatiformes	
Urolophidae	
Urolophus aurantiacus	2
Myliobatididae	
Myliobatis tobijei	1
Chimaeriformes	
Chimaeridae	
Chimaera phantasma	1
Hydrolagus mitsukurii	2
Rhinochimaeridae	
Rhinochimaera pacifica	1

External features of sperm. The sperm of 35 species consisted of a head including an acrosome, a midpiece and a slender flagellum. The total length of the sperm ranged from 93 μm in *Galeocerdo cuvier* to 224 μm in *S. japonica* (Table 2). The external features of the head and midpiece of the sperm varied in each species (Fig. 1C-H). The head in most species was helical, but in *C. anguineus*, *Dalatias licha*, three species of the genus *Etmopterus*, and *S. japonica*, the sperm in the ampulla did not display a clear helical form in the head (Fig. 1F). The tip of the head was bent like a gaff (Fig. 1C, H). The number of gyres of the sperm with the helical form ranged from 3 to 24 (Table 2). The head length in elasmobranchs was more than 30 μm, while that in holocephalans was less than 25 μm. The longest sperm head was 93 μm in *Centroscymnus owstoni*. The standard deviation of the head length in each species was 1.03 to 2.91 μm. The proportion of the head to the total length ranged from 7 to 45 % (Table 2).

The midpiece was much shorter than the head in elasmobranchs. In contrast, two species of Chimaeridae had a long midpiece compared to the head. The width of the midpiece was slightly thicker than the head (Fig. 1C-H). The midpiece length ranged from 6 to 21 μm, and the

Source : MNHN, Paris

TABLE 2. — Summary of the measurement and condition of the sperm in 35 chondrichthyan fishes.
Type of sperm aggregate: 1, Solitary or sperm clumps, 2, Single-layer spermatozeugmata, 3, Compound spermatozeugmata, 4, Spermatophores (rice-grain type), 5, Spermatophores (atypical rod). Values in parentheses indicate a ratio to total length.

Species	Code in Fig. 2	Type of sperm aggregate	Number of gyres	Total length in µm	Head length in µm (ratio)	Midpiece length in µm (ratio)	Flagellum length in µm (ratio)
Heterodontus japonicus	HDJ	1	6	156	41 (26)	14 (9)	102 (65)
Orectolobus japonicus	OLJ	3	5	136	49 (36)	14 (10)	73 (54)
Carcharias taurus	CCT	5	17	110	37 (34)	13 (12)	60 (54)
Alopias pelagicus	APP	4	12	100	37 (37)	9 (9)	54 (54)
Isurus oxyrinchus	IRO	4	7	134	60 (45)	12 (9)	62 (46)
Cephaloscyllium umbratile	CSU	2	24	186	48 (26)	21 (12)	116 (62)
Galeus eastmani	GLE	2	13	197	45 (23)	19 (10)	133 (67)
Galeus nipponensis	GLN	2	14	197	44 (22)	19 (10)	134 (68)
Hemitriakis japanica	HTJ	2	23	120	46 (38)	14 (12)	60 (50)
Mustelus canis	MLC	2	11	98	41(42)	7 (7)	50 (51)
Mustelus griseus	MLG	2	10	114	47 (41)	9 (8)	58 (51)
Mustelus manazo	MLM	2	12	122	38 (31)	10 (8)	74 (61)
Carcharhinus plumbeus	CRP	2	7	101	33 (33)	9 (9)	59 (58)
Galeocerdo cuvier	GCC	2	17	93	35 (38)	9 (10)	49 (52)
Prionace glauca	PNG	3	6	116	34 (30)	9 (7)	73 (63)
Sphyrna lewini	SRL	3	12	109	41 (38)	8 (7)	60 (55)
Chlamydoselachus anguineus	CLA	1	0	175	46 (26)	19 (11)	110 (63)
Centroscymnus owstoni	CTO	1	23	210	93 (44)	20 (10)	97 (46)
Dalatias licha	DTL	1	0	130	50 (38)	12 (10)	68 (52)
Deania calcea	DNC	1	3	141	56 (40)	13 (9)	72 (51)
Deania historicosa	DNH	1	3	162	55 (34)	15 (9)	92 (57)
Etmopterus brachyurus	EPB	1	0	193	63 (33)	16 (8)	114 (59)
Etmopterus molleri	EPM	1	0	178	58 (33)	16 (8)	104 (59)
Etmopterus pusillus	EPP	1	0	172	49 (28)	17 (10)	106 (62)
Squalus brevirostris	SLB	1	10	166	36 (22)	11 (7)	119 (71)
Squalus japonicus	SLJ	1	10	153	35 (23)	9 (6)	109 (71)
Squatina japonica	STJ	1	0	224	64 (29)	16 (7)	143 (64)
Rhinobatos schlegelii	RBS	1	6	131	46 (35)	9 (7)	76 (58)
Torpedo tokionis	TPT	2	5	130	51 (39)	10 (8)	69 (53)
Raja eglanteria	RJE	2	6	130	39 (30)	17 (13)	74 (57)
Urolophus aurantiacus	UPA	1	7	129	55 (44)	11 (8)	63 (48)
Myliobatis tobijei	MBT	1	5	96	35 (36)	10 (11)	51 (53)
Chimaera phantasma	CMP	1	3	137	11 (8)	11 (8)	115 (84)
Hydrolagus mitsukurii	HLM	1	3	164	11 (7)	18 (11)	135 (82)
Rhinochimaera pacifica	RCP	1	3	112	20 (18)	6 (5)	86 (77)

disparities among species were small. The standard deviation of the midpiece length in each species was 0.37 to 1.71 µm. The flagellum length ranged from 49 µm in *G. cuvier* to 143 µm in *S. japonica*. The standard deviation of the flagellum length was 1.42 to 3.45 µm. In *Isurus oxyrinchus*, *C. owstoni* and *Urolophus aurantiacus*, the flagellum was almost the same length as the head. The proportion of the flagellum in Chimaeriformes was more than 75%.

Source : MNHN, Paris

Numerical taxonomic analysis.

The cluster analysis indicated the similarity of external features of the sperm within the genus and/or family (Fig. 2). The sperm of the two species in *Galeus* and *Squalus* were similar, while the similarity in two species of *Deania* was low compared to the former genera. In *Etmopterus* and *Mustelus*, the sperm of two of the three species were much alike, but that of each remaining species was similar to that of other genera. The external features of the sperm in *Squalus* spp. were very different from those in the other genera of Squalidae. The similarity of the families, except Scyliorhinidae, within the Carcharhiniformes was almost equal to that of the genera, except *Squalus*, within the Squalidae. The cluster analysis also divided the 35 species into 4 groups (Fig. 2).

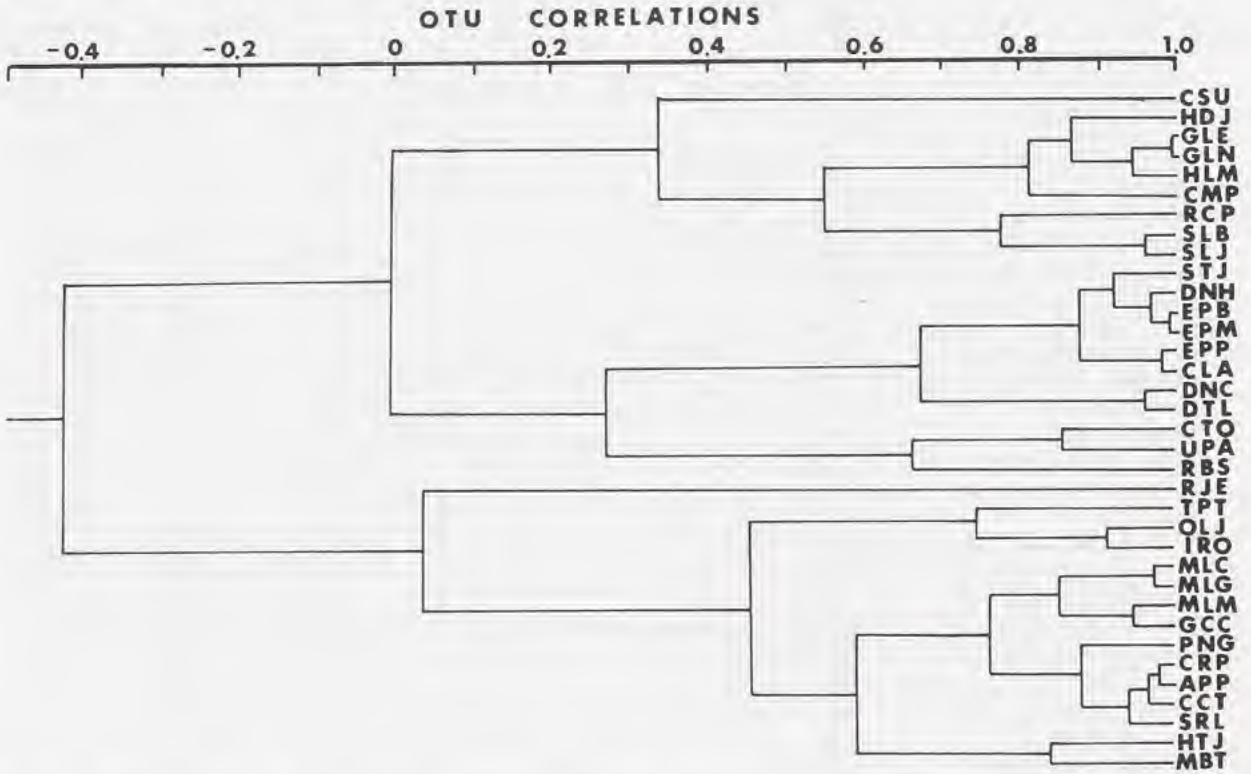

FIG. 2. — Phenogram of 35 species in chondrichthyan fishes, using six characters of sperm morphology in cluster analysis. Three letter codes at right indicate species name (see Table 2). Cophenetic correlation coefficient: 0.820.

Group I CSU to SLJ. This group included four orders, five families; Scyliorhinidae of the Carcharhiniformes, Heterodontiformes, two families of Chimaeriformes, and Squaliformes. Only the Scyliorhinidae was distant from the other families of Carcharhiniformes. The sperm of three species of Scyliorhinidae were much longer than those of the other species of Carcharhiniformes. *Cephaloscyllium umbratile*, in which the sperm had 24 gyres, differed from the other species in the group. The proportion of the head and flagellum in *Squalus* spp. was smaller and larger than that in the other genera of Squaliformes, respectively.

Group II STJ to RBS. This group consisted of five orders, five families; Squatiniformes, Squaliformes, Hexanchiformes, Urolophidae of Myliobatiformes, and Rhinobatiformes. Most of the species of Squalidae belonged to the group. Urolophidae was separated from Myliobatididae of the same order. The group was divided into two subgroups. The number of the gyres of the

Source : MNHN, Paris

sperm in the subgroup from STJ to DTL was less than that in the subgroup from CTO to RBS. The sperm of *C. owstoni*, alone in the Squalidae, had a large number of gyres.

Group III RJE. This group contained only one species, *Raja eglanteria*. The proportion of the midpiece in this species was large compared to the other species, while the sperm had a small number of gyres.

Group IV TPT to MBT. This group included five orders and nine families; Torpediniformes, Orectolobiformes, three families of Lamniformes, three families of Carcharhiniformes, and the Myliobatididae in the Myliobatiformes. All species of Carcharhiniformes except Scyliorhinidae belonged to this group. The group was divided into two subgroups. The sperm in the subgroup from TPT to IRO was longer than that in another subgroup. Only *I. oxyrinchus* was separated from the other species of Lamniformes. In the Triakidae, *Hemitriakis japanica* was separated from the other species. The sperm of *H. japanica* had a larger number of gyres than other species of the same family.

DISCUSSION

The internal morphology of the sperm, especially the structure of the flagellum, displays differences between elasmobranchs and holocephalans [4, 6, 8]. However, literature which deals with the relationship between the external features of the sperm and the phylogeny in chondrichthyan fishes is scanty. The external features of the sperm in chondrichthyan fishes were recognized to be species specific. They showed a similarity within the genus and/or family. Four groups based on the cluster analysis also suggested a similarity of sperm morphology within the order. COMPAGNO [1] and SHIRAI [12] divided elasmobranchs into four and two groups, respectively. Both authors recognized the four orders Heterodontiformes, Orectolobiformes, Lamniformes and Carcharhiniformes, as one group. The present study also recognized one group consisting of three orders excepting the Heterodontiformes. The remaining three groups of COMPAGNO: 1. Hexanchiformes, Squaliformes and Pristiophoriformes, 2. Batoids (Skates and Rays), and 3. Squatiniformes, are equal to the group 2 of SHIRAI. COMPAGNO [1] considered that the three groups are independently derived, while SHIRAI [12] regarded them as one of the two groups derived from a basal group. Group II in the present study includes five orders and is close to SHIRAI's grouping. COMPAGNO [2] divided Carcharhiniformes into two suborders; Scyliorhinoidei and Carcharhinoidei. The Scyliorhinidae of Group I belongs to the former, and the three families of Group IV belong to the latter.

COMPAGNO [2]: 4 groups	SHIRAI [12]: 2 groups
1. Heterodontiformes Orectolobiformes Lamniformes Carchariniformes	Group 1
2. Hexanchiformes Squaliformes Pristiophoriformes	Group 2
3. Batoids	
4. Squatiniformes	

Source : MNHN, Paris

The families of Group I, except two species of Squalidae, and only one family, Rajidae, of Group III are oviparous [3]. The formation of sperm aggregates has been demonstrated in various species of elasmobranchs [11]. In this study, it was found to be of the same type within the order. This may be related to the similarity of reproductive modes within the order [3]. Thus, the grouping of chondrichthyan fishes based on the external features of the sperm in this account reflects the systematics and phylogeny derived from consideration of the external, skeletal and muscular systems of the body and the female reproductive modes.

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Source : MNHN, Paris

Comparative Spermatology of Anurans with Special References to Phylogeny

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ABSTRACT

The anurans so far examined can be divided into three groups based on the data of comparative spermatology. The first group, Ascaphidae and Discoglossidae, is considered to be plesiomorphic because it has most plesiomorphic characters, endonuclear canal, rod-shaped endonuclear perforatorium and mitochondria adjacent to only the axial rod as in urodeles. The second group, including Myobatrachidae, Bufonidae, Hylidae and Leptodactylidae is characterized by a conical acrosome and a conical extranuclear perforatorium. However, Myobatrachidae differ from the other three families, in mitochondrial position and centriolar arrangement. These characteristics are rather similar to those of ascaphids, discoglossids and urodeles. Thus Myobatrachidae appears to be the most primitive among this group. The tail of the first two groups consists of an axoneme, axial rod and undulating membrane. However it contains an axoneme and axial rod in *Hyla*, and an axoneme only in *Telmatobius*. The third group including Pipidae, Ranidae and Rhacophoridae is characterized by a 140° angle of centrioles and a tail with only the axoneme. These features might represent a synapomorphy for this group. The main evolutionary pathways observed in anurans are: 1) a reduction in the length of the endonuclear canal and the eventual disappearance of this structure; 2) the disappearance of the subacrosomal cone and the perforatorium; 3) the disappearance of the undulating membrane and the axial rod; and 4) an increase in the angle between the two centrioles. Phylogenetic conclusions drawn from sperm ultrastructure coincide in many points with previous data based on somatic features.

RÉSUMÉ

Spermatologie comparée des Anoures et rapports avec la phylogénie

Les Anoures examinés jusqu'ici peuvent être divisés en trois groupes en fonction de la spermatologie comparée. Le premier groupe, les Ascaphidae et Discoglossidae, est considéré comme plésiomorphe parce qu'il possède la plupart des caractères plésiomorphes: canal endonucléaire, perforatorium endonucléaire en forme de baguette et mitochondrie adjacente seulement à la baguette axiale, comme chez les Urodèles. Le second groupe, comprenant les Myobatrachidae, Bufonidae, Hylidae et Leptodactylidae est caractérisé par un acrosome conique et un perforatorium extranucléaire conique. Toutefois, les Myobatrachidae diffèrent des trois autres familles par la position de la mitochondrie et la disposition des centrioles. Ces caractéristiques sont assez proches de celles des Ascaphidae, Discoglossidae et Urodèles. Les Myobatrachidae semblent donc être les membres les plus primitifs de ce groupe. La queue, dans les deux premiers groupes, consiste en un axonème, une baguette axiale et une membrane ondulante. Toutefois, elle contient seulement un axonème et une baguette axiale chez *Hyla*, et seulement un axonème chez *Telmatobius*. Le troisième groupe, comprenant les Pipidae, Ranidae et Rhacophoridae, est caractérisé par un angle de 140° des centrioles et une queue contenant seulement l'axonème. Ces caractéristiques pourraient représenter une synapomorphie pour ce groupe. Les étapes évolutives principales observées chez les Anoures sont 1) une réduction de la longueur du canal endonucléaire et la disparition finale de cette structure; 2) la disparition du cône subacrosomien et du perforatorium; 3) la disparition de la membrane ondulante; et 4) l'augmentation de l'angle entre les deux centrioles. Les conclusions phylogénétiques tracées à partir de l'ultrastructure des spermatozoïdes coïncident en de nombreux points avec les données précédentes basées sur les caractéristiques somatiques.

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Source : MNHN, Paris

Amphibia are divided into the three orders Apoda, Urodela and Anura. The Anura is often considered as a super-order [30]. Anura can be subdivided into two distinct levels of organization, with primitive and higher families, on morphological data. However, the phyletic relationships between and within the two levels are extremely difficult to clarify because anurans are morphologically quite conservative.

The ultrastructure of the spermatozoon has been the subject of considerable study in the past twenty years in several animal groups and is recognized as an important indicator of phylogenetic relationships [6, 15, 21, 28, 31]. Anuran spermatozoa provide a useful suite of taxonomic characters because they have sufficient variety in the structure of the acrosome, perforatorium, and tail, and the arrangement of the centrioles. Despite the taxonomic value of sperm structure, there have been little data available on phylogeny of anuran spermatozoa.

The ultrastructure of the spermatozoon is now known in 31 species (17 genera) of anurans [12-13, 18, 22-27, 29, 34-39]. This enables us to give a summary of the ultrastructure of the spermatozoon and its phylogeny.

The purpose of this chapter is to describe the ultrastructure of spermatozoa in six species of four families of anurans and to compare it to those of other groups of amphibians. The results are also discussed with regard to the phylogenetic position.

MATERIALS AND METHODS

Spermatozoa from the following anuran species were used in this study (asterisks indicate new data); *Ascaphus truei* (Ascaphidae); *Discoglossus pictus, Alytes obstetricians, Bombina variegata, *B. orientalis* (Discoglossidae); *Adelotus brevis, Limnodynastes peronii, Mixophyes fasciolatus, Neobatrachus pelobatiodes* (Myobatrachidae); *Bufo arenarum, B. marinus, *B. bufo gargarizans* (Bufonidae); *Odontophrynus cultripes, Telmatobius hauthali* (Leptodactylidae); *Litoria caerulea, L. fallax, L. gracilenta, L. lesueuri, L. peronii, L. rubella, Pachymedusa dacnicolor, *Hyla japonica, H. meridionalis* (Hylidae); *Xenopus laevis* (Pipidae); *Rana clamitans, R. pipiens, *R. nigromaculata, *R. dybowskii, *R. rugosa* (Ranidae); *Rhacophorus arboreus, R. schlegelii* (Rhacophoridae).

Species of anurans were collected in the neighbourhood of Taegu, Korea. Testes were dissected and fixed in 2.5%-5% glutaraldehyde in 0.1M sodium cacodylate buffer and post-fixed in 1% osmium tetroxide in the same buffer. They were then dehydrated in a graded ethanol series and embedded in Epon 812. The samples were sectioned on a Sorvall MT 2-B ultramicrotome, stained in 4% aqueous uranyl acetate, post-stained with lead citrate and examined with a Hitachi H-600 electron microscope.

OBSERVATIONS

Bombina orientalis

B. orientalis possesses a spermatozoon with a peculiar architecture unlike the classical sequence of acrosome, nucleus and flagellum. The flagellum is juxtaposed longitudinally along the nucleus. The nucleus is cone shaped and some nuclear lacunae, irregular in shape, are scattered within the nucleus. The chromatin is not completely compact but condensed into large masses (Fig. 1a).

The acrosome consists of a thin vesicle truncated at the anterior end and its material is homogenous and moderately electron dense. The subacrosomal space contains the perforatorium

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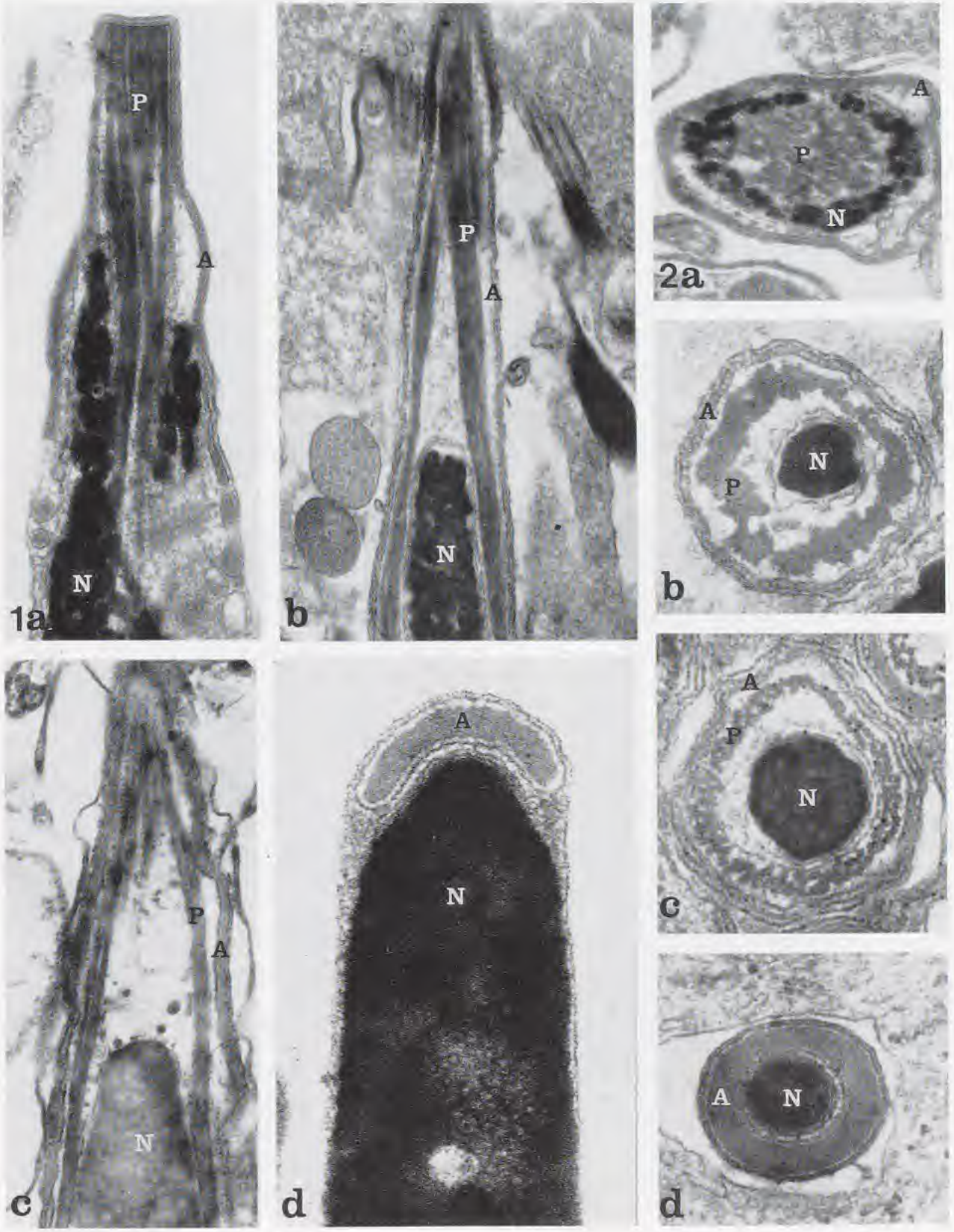
FIG. 1. — Longitudinal sections of head. **a:** *Bombina orientalis*, x 30 000; **b:** *Bufo bufo gargarizans*, x 35 700; **c:** *Hyla japonica*, x 44 000; **d:** *Rana dybowskii*, x 75 000.

FIG. 2. — Transverse sections of head. **a:** *Bombina orientalis*, x 30 000; **b:** *Bufo bufo gargarizans*, x 56 000; **c:** *Hyla japonica*, x 63 000; **d:** *Rana rugosa*, x 52 000.

Abbreviations used in the figures: A, acrosome; AR, axial rod; Ax, axoneme; C, centriole; EC, endonuclear canal; FS, fibrogranular sheet; M, mitochondria; MF, marginal filament; N, nucleus; P, perforatorium; PM, pericentriolar material; UM, undulating membrane; V, vesicle.

Source : MNHN, Paris



Source : MNHN, Paris

which emerges from an endonuclear canal in the tip of the nucleus (Figs 2a, 3a). The perforatorium consists of bundles of filamentous material arranged parallel to each other.

The neck region is located in the lateral surface of the nucleus which is furrowed by a longitudinal depression which contains an anterior portion of the axial rod and proximal centriole (Fig. 3a, b). Two centrioles form an angle of 70°, approximately, to each other and the proximal centriole is perpendicular to the longitudinal axis.

The tail contains an axoneme, axial rod, undulating membrane and mitochondria. The axoneme, of the typical 9+2 pattern, is lateral to the main axis which is occupied by the axial rod. The axial rod extends almost to the tip of the flagellum. The undulating membrane is short in the proximal region of the tail and becomes longer toward the middle region. Mitochondria are arranged in a semicircle and positioned only around the axial rod (Fig. 4a).

Bufo bufo gargarizans

The nucleus is cylindrical in shape and the chromatin is completely condensed. The base of the nucleus holds an implantation fossa in which the proximal centriole lodges.

The acrosome consists of a very thin vesicle containing homogenous material of moderate electron density as in *B. orientalis* (Fig. 1b). The subacrosomal space contains the perforatorium consisting of bundles of microfilaments. The perforatorium runs along the inner acrosomal membrane, unlike *B. orientalis*(Fig. 2b). An endonuclear canal is not present in this species.

In the neck region two centrioles are perpendicular to each other and embedded in pericentriolar material (Fig. 3c). The pericentriolar material connecting with the anterior end of the axial rod shows transverse striations.

The tail contains an axoneme, axial rod, undulating membrane and mitochondria. The axial rod which is associated with axonemal doublet no. 3 extends to the principal piece of the flagellum (Fig. 5a). The end piece contains only the axoneme. Mitochondria constituting a sheath are separated from the flagellum by the cytoplasmic canal and surround the axoneme, axial rod and undulating membrane (Fig. 4b).

Hyla japonica

The spermatozoon of *H. japonica* has an ultrastructure very similar to that of *B. bufo gargarizans* (Figs 1c, 3d). Differences between two will be noted here.

The spermatozoa are shorter and more slender than those of *B. bufo gargarizans*. The perforatorium consists of microtubule-like arrays instead of the microfilaments in other anuran spermatozoa (Fig. 2c). No transverse striations appear in the pericentriolar material.

The tail contains an axoneme and axial rod without undulating membrane. The axial rod is closer to the axonemal doublet no. 3 in the middle and principal pieces (Figs 4c, 5b) but disappears in the distal region of the flagellum.

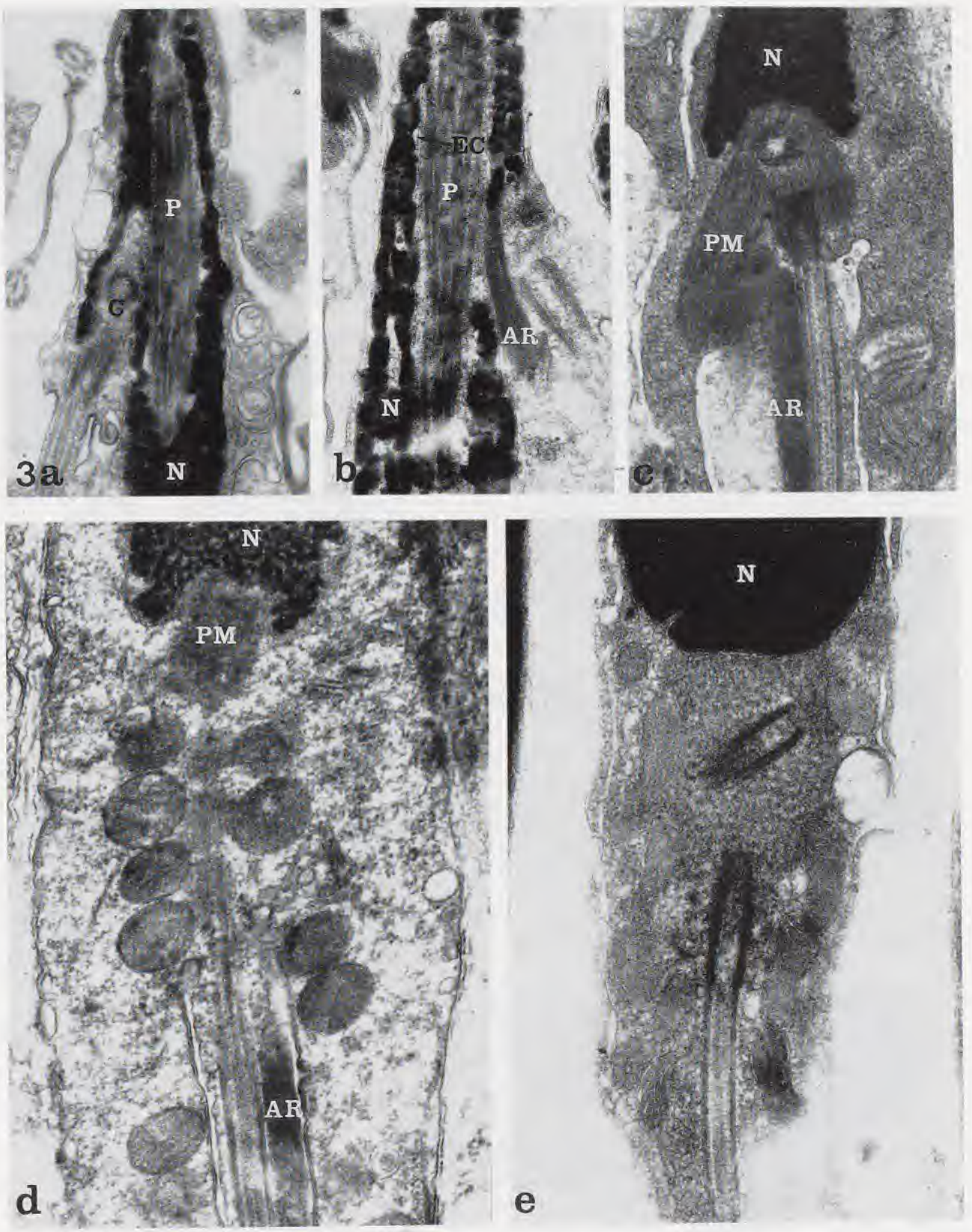
Rana nigromaculata, Rana dybowskii, Rana rugosa

The spermatozoa of all three species examined have a very similar ultrastructure except the pericentriolar material.

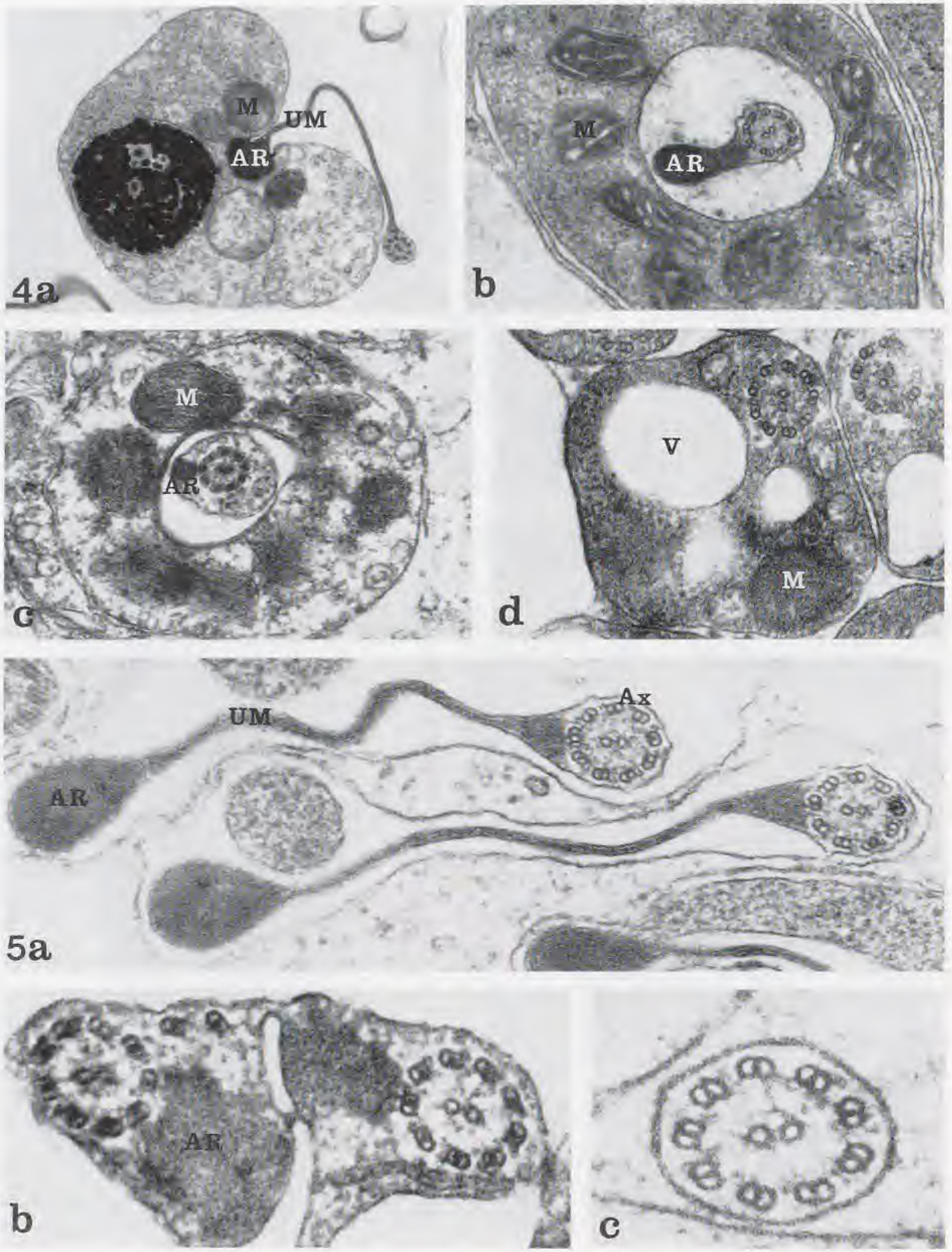
The nucleus, with compact chromatin, is cylindrical and slightly tapered at both ends. The posterior end of the nucleus has an implantation fossa in which the centrioles do not reside. Intranuclear inclusions are visible within the nucleus.

FIG. 3. — Longitudinal sections of head-tail junction. **a,** *Bombina orientalis*, x 23 000; **b:** *Bombina orientalis*, x 30 000; **c:** *Bufo bufo gargarizans*, x 34 000; **d:** *Hyla japonica*, x 30 000; **e:** *Rana rugosa*, x 36 000.

Source : MNHN, Paris



Source : MNHN, Paris



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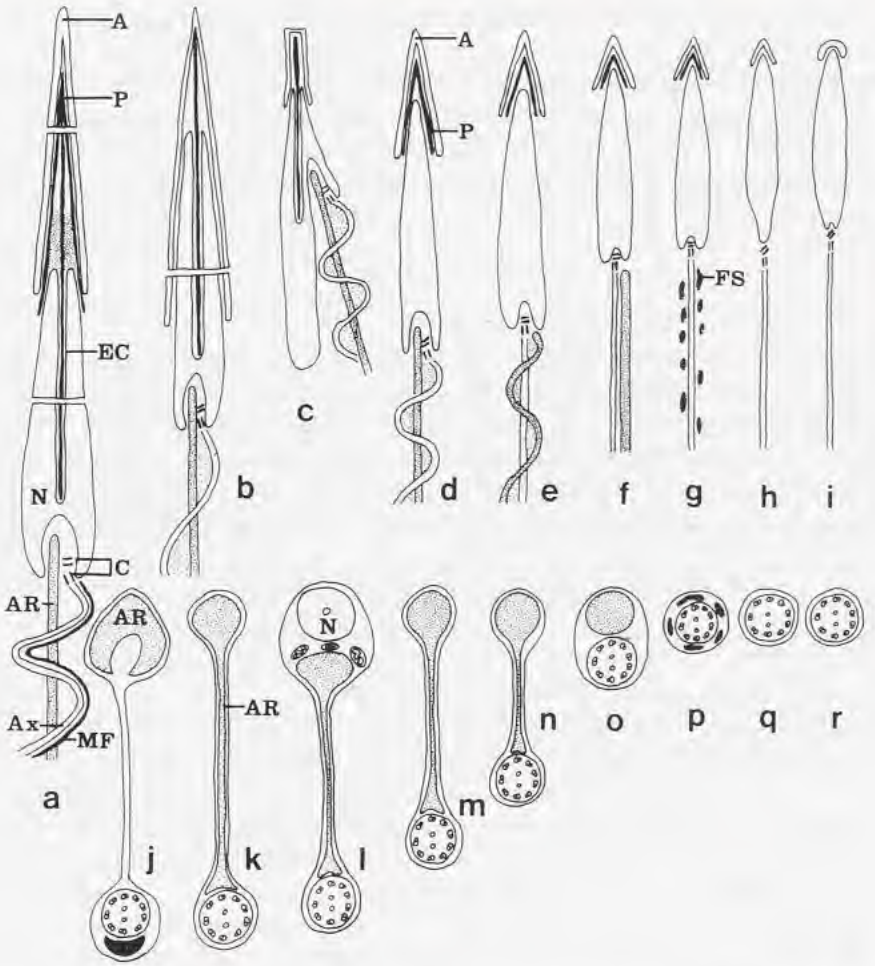

FIG. 6. — Diagrammatic representation of sections of amphibian spermatozoa. **a-i:** longitudinal sections; **j-r:** cross sections of the principal piece. **a, j:** Salamandridae, *Pleurodeles* [32]. **b, k:** Discoglossidae, *Discoglossus* [39]. **c, l:** Discoglossidae, *Bombina* [23]. **d, m:** Myobatrachidae, *Limnodynastes* [25]. **e, n:** Leptodactylidae, *Odontophrynus* [12]. Bufonidae, *Bufo* [24]. Hylidae, *Pachymedusa* [37], Hylidae, *Litoria* [26]. **f, o:** Hylidae, *Hyla* [27]. **g, p:** Leptodactylidae, *Telmatobius* [34]. **h, q:** Pipidae, *Xenopus* [13]. **i, r:** Ranidae, *Rana*.

The acrosome is a saclike structure situated at the most anterior portion of the nucleus (Fig. 1d). It is shorter and thicker than in other species mentioned above. There is no perforatorium nor an endonuclear canal (Figs 1d, 2d).

The neck region contains the centrioles, pericentriolar material and mitochondria (Fig. 3e). Two centrioles form an angle of approximately 140° and the distal centriole is parallel to the main axis. Two centrioles are situated outside the nuclear fossa. Mitochondria aggregate around the base of nucleus, the centrioles and the axoneme. A cytoplasmic canal is not observable.

FIG. 4. — Transverse sections of middle piece. **a:** *Bombina orientalis*, x 21 000; **b:** *Bufo bufo gargarizans*, x 42 000; **c:** *Hyla japonica*, x 40 000; **d:** *Rana dybowskii*, x 70 000.

FIG. 5. — Transverse sections of principal piece. **a:** *Bufo bufo gargarizans*, x 72 000; **b:** *Hyla japonica*, x 10 800; **c:** *Rana nigromaculata*, x 145 000.

Source : MNHN, Paris

The tail contains a 9+2 pattern axoneme without axial rod and undulating membrane (Figs 4d, 5c).

The spermatozoa of the three species have some differences in the neck region. In *R. rugosa*, the pericentriolar material surrounds the centrioles and contains transverse striations (Fig. 3e). However, in *R. dybowskii*, it is only located lateral to the centriole and also has transverse striations. In *R. nigromaculata,* no transverse striations are observed within the pericentriolar material surrounding the centrioles.

DISCUSSION

Comparative ultrastructure of spermatozoa in anurans.

The ultrastructure of spermatozoa is described in six species from four genera of four families (Discoglossidae, Bufonidae, Hylidae and Ranidae) of anurans in this chapter. *B. orientalis*, *B. bufo gargarizans* and *H. japonica* have spermatozoa that conform to the generalized amphibian sperm (see JAMIESON, this volume). On the other hand, *Rana* species have spermatozoa that differ markedly from the above anurans in their organization.

The acrosome is conical in most anurans, but is saccular in *Rana*, and is a coiled structure to one side of the nucleus in *Rhacophorus* [29]. This character of the saccular and coiled acrosome is considered to be apomorphic as compared with the conical acrosome.

The subacrosomal cone present in the sperm of *Ascaphus* [22] is characteristic of urodeles [32, 33] but is not present in any other anurans. JAMIESON *et al.* [22] reported that this structure is a widespread and plesiomorphic feature of amniote sperm.

The endonuclear canal as a main characteristic structure of urodelan sperm has been observed in the primitive anuran families, Ascaphidae [22] and Discoglossidae [18, 23, 39]. This character should be considered as plesiomorphic compared to the other anurans which lack the canal. *Discoglossus* [39] especially has a long canal running the entire length of the nucleus as in the urodeles. In *Bombina* and *Alytes* [18] the canal penetrates roughly to the middle of the nucleus. The perforatorium has been observed in many anurans [12, 14, 18, 22-27, 34, 36-39] and urodeles [8-11, 31-32] but shows differences in its shape and position. The ascaphids [22] and discoglossids [18, 23, 39] have a rod-shaped endonuclear perforatorium which is also characteristic of urodeles. This must be considered plesiomorphic, in comparison with the completely conical extranuclear perforatorium which has been observed in myobatrachids [25], bufonoids [14, 24, 26], hylids [26-27, 37] and leptodactylids [12, 34]. The higher anuran families, Pipidae, Ranidae and Rhacophoridae have no perforatorium [13, 29, 35, 38]. It can thus be deduced that absence of the perforatorium is an apomorphic character in anurans. The perforatorium is progressively simplified and finally disappears in several higher arthropods and vertebrates during evolution of a terrestrial life [6].

The orientation of the centrioles varies depending on the systematic position of the animal [1]. Anurans show three types of orientation of the centrioles. In the first type the distal and proximal centrioles lie at an angle of 70° to each other whereas the proximal centriole and the main axis are perpendicular to each other. This type has been observed in ascaphids [22], discoglossids [18, 23, 39] and myobatrachids [25]. In the second type the two centrioles are perpendicular to each other. This type is seen in bufonoids [14, 24], hylids [27, 37] and leptodactylids [12, 34]. The third type is characterized by the centrioles forming an angle of approximately 140° to each other. This type is seen in pipids [13, 38] and ranids [35].

The tail has been used as an important taxonomic character in many animal groups [1-5, 7, 16, 28]. The tail of most amphibians contains an axoneme, axial rod and undulating membrane. In pipids [13, 38], ranids [35], rhacophorids [29] and the leptodactylid *Telmatobius* [34] the tail has an axoneme only. *Hyla* in the Hylidae [27, 36] has a tail in which the axoneme and axial rod lack an undulating membrane. This tail seems to be the intermediate type in anuran sperm. We thus conclude that a tail consisting of undulating membrane and axial rod is plesiomorphic.

Source : MNHN, Paris

The position of the mitochondria varies in anuran sperm. Mitochondria adjacent to only the axial rod have been observed in ascaphids (termed a paraxonemal rod in this species) [22], discoglossids [18, 23, 39], myobatrachids [25] and urodeles [8-11, 32-33]. In bufonoids [14, 24, 26], hylids [26-27, 37] and leptodactylids [12, 34], they surround the axoneme and constitute a sheath separated by the cytoplasmic canal. The mitochondrial sheath without cytoplasmic canal is observed in pipids [13, 38] and ranids [34]. LEE & JAMIESON [25] suggested in myobatrachids that the location of mitochondria adjacent to only the axial rod is plesiomorphic as compared with the mitochondria surrounding the axoneme.

Phylogenetic relationships in anurans based on spermatozoal ultrastructure.

Table 1 shows phylogenetic trends within the anurans, based on the data of comparative spermatology. The main evolutionary tendencies observed in anurans based on ultrastructural characteristics of spermatozoa are (1) the disappearance of the subacrosomal cone of Ascaphidae in the other anuran groups, (2) a reduction in the length of the endonuclear canal relative to Ascaphidae and Discoglossidae and the eventual disappearance of this structure from the other anuran groups, (3) the disappearance of the perforatorium and the axial rod in Pipidae and

TABLE 1. — Phylogenetic relationships based on the ultrastructure of anuran spermatozoa
PC, plesiomorphic characters; AC, apomorphic characters; Ur, Urodeles; As, Ascaphidae; Di, Discoglossidae; My, Myobatrachidae; Bu, Bufonidae; Hy, Hylidae; Le, Leptodactylidae; Pi, Pipidae; Ra, Ranidae; Rh, Rhacophoridae. *, no data.

	families	Ur	As	Di	My	Bu	Hy	Le	Pi	Ra	Rh
	genera		*Ascaphus*	*Discoglossus Alytes Bombina*	*Limnodynastes Mixophyes Neobatrachus*	*Bufo*	*Pachymedusa Litoria Hyla*	*Odontophrynus Telmatobius*	*Xenopus*	*Rana*	*Rhacophorus*
	characters										
PC	marginal filament										
PC	axial rod displaced from axoneme										
PC	subacrosomal cone										
PC	ring structure		*								
PC	endonuclear canal										
PC	endonuclear perforatorium										
PC	45°-70° angle of centrioles										
PC	mitochondrial location related to axial rod										
PC	undulating membrane										
PC	axial rod										
PC	extranuclear perforatorium										
PC	conical acrosome										
AC	sperm tail with axoneme only										
AC	140° angle of centrioles										*
AC	saccular or lateral acrosome										
AC	helical spermatozoa										

Source : MNHN, Paris

Ranidae, (4) the disappearance of the undulating membrane in Hylidae, Pipidae and Ranidae, (5) an increase in the angle between the two centrioles: 30°-70° in Ascaphidae, Discoglossidae and Myobatrachidae, 90° in Leptodactylidae, Bufonidae and Hylidae, 140° in Pipidae and Ranidae (Fig. 6).

Anurans studied so far can be divided into three groups with regard to phylogenetic relationships based on the ultrastructure of spermatozoa. The first group includes Ascaphidae and Discoglossidae. They appear drastically isolated from the other anurans because they are characterized by an endonuclear canal, a rod-shaped endonuclear perforatorium, the angle of the centrioles and location of mitochondria adjacent to only the axial rod. The subacrosomal cone appears in only Ascaphidae. These characters show most of the common feature of urodeles. Therefore this group should be considered as plesiomorphic and the Ascaphidae as more plesiomorphic than the Discoglossidae. This interpretation perfectly agrees with the traditional classification. Based on the ultrastructure of acrosome and incompletely condensed nucleus, *Bombina* and *Alytes* are more closely related than they are to *Discoglossus*; *Discoglossus* [39] has the ring structure and the endonuclear canal occupying the whole length of the nucleus which has also been observed in urodeles. With respect to the above characteristics, *Discoglossus* seems to be the most primitive genus among Discoglossidae. However, this interpretation disagrees with the phylogenetic position of three genera from anatomical and karyological data. *Bombina* is the most primitive in an osteological classification [40]. *Alytes* is the most primitive from the karyological point of view in having a karyotype similar to that of primitive urodeles [30].

The second group, including Myobatrachidae, Bufonidae, Hylidae and Leptodactylidae, is characterized by common features: the conical acrosome and the conical extranuclear perforatorium. Myobatrachidae are quite different from this group in having the same structure of the sperm tail as the Ascaphidae and Discoglossidae. These characteristics suggest that Myobatrachidae may occupy the most primitive position in this group. Myobatrachidae has been classified as a subfamily of the family Leptodactylidae. However, recent phylogenies using morphological data [17] or molecular data [20] have recognized myobatrachids as a family separate from the Leptodactylidae. Ultrastructural data of the spermatozoa are in agreement with the latter interpretation [25]. Bufonidae, Hylidae and Leptodactylidae are also united by a single apomorphy, mitochondrial location surrounding the axoneme. This contrasts with the plesiomorphic location of the mitochondria associated with the axial rod in ascaphids [22], discoglossids [18, 23, 39] and urodeles [8-11, 32-33]. The phyletic affinity between these three families is recognized by various authors [19-20, 30].

The third group includes Pipidae, Ranidae and Rhacophoridae. They have common features, such as no perforatorium and a tail with only the axoneme. Although they are different in the morphology of the acrosome, Pipidae and Ranidae are closely related based on the centriolar arrangement as well as the above two characters. The conical acrosome of Pipidae appears as a plesiomorphic character in comparison to the saccular acrosome in Ranidae. This relationship between two families coincides with the morphological data suggested by GRIFFITHS [19]. However, according to MORESCALCHI [30], Pipidae are closely related to the Discoglossidae from karyological data. Rhacophoridae are characterized by the coiled acrosome lying on one side of nucleus.

In anurans the classification based on comparative spermatology is almost similar to traditional classification. Comparative spermatology thus may be considered as a useful new tool for the understanding of anuran phylogeny.

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Source : MNHN, Paris

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Source : MNHN, Paris

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Source : MNHN, Paris

Amphibian Sperm: Phylogeny and Fertilization Environment

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ABSTRACT

Sperm characteristics such as the structure and position of the acrosome, the design and ultrastructure of the flagellum, the presence/absence of a neck-piece and several other fine structural details are important diagnostic features which distinguish the three orders, families and species of lissamphibians. These sperm features are also important in phylogenetic inferences. It appears that the axoneme-undulating membrane-axial rod represents a plesiomorphic character. This pattern has been simplified to a single axoneme as a secondary reversal for external fertilization in many anurans or has developed as a double axoneme as in *Chiromantis xerampelina*. Sperm head and acrosome length are predictive in distinguishing between terrestrial and aquatic anuran fertilizers. Sperm motility is species specific among representatives of seven of the nine South African families and motile sperm which exhibit forward progression are the rule for aquatic fertilizing anurans. In contrast, the sperm of terrestrial fertilizing anurans are immotile in a wide range of physiological/culture media with osmotic concentrations varying from 10 to 300 mOsm/kg. The term ect-terrasperm is suggested as a new terminology for amphibians which exhibit the terrestrial mode of fertilization.

RÉSUMÉ

Spermatozoïdes des Amphibiens: phylogénie et environnement de la fécondation

Les caractéristiques du spermatozoïde telles que la structure et la position de l'acrosome, la forme et l'ultrastructure du flagelle, la présence ou absence d'un cou et plusieurs autres détails ultrastructuraux sont des critères de diagnostic qui distinguent les trois ordres, les familles et les espèces de Lissamphibiens. Les caractéristiques du spermatozoïde sont aussi importantes pour la compréhension de la phylogénie. Il semble que l'axonème avec membrane ondulante et fibre axiale représente un caractère plésiomorphe. Cette structure a été simplifiée en un axonème simple, comme réversion secondaire pour la fécondation externe, chez de nombreux Anoures, ou s'est développée en un axonème double comme chez *Chiromantis xerampelina*. La longueur de la tête du spermatozoïde ou de l'acrosome permet de distinguer de manière prédictive les Anoures à fécondation aquatique ou terrestre. La motilité du spermatozoïde est spécifique des espèces parmi les représentants de sept parmi les neuf familles d'Afrique du Sud, et les spermatozoïdes mobiles qui montrent une progression vers l'avant sont la règle pour les Anoures à fécondation aquatique. Par contre, les spermatozoïdes des Anoures à fécondation terrestre sont immotiles dans de nombreux milieux physiologiques et de culture avec des concentrations osmotiques variant de 10 à 300 mOsm/kg. Le terme ect-terraspermatozoïde est suggéré comme une nouvelle terminologie pour les Amphibiens qui possèdent le mode terrestre de fécondation.

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Source : MNHN, Paris

Studies at both the light microscopic and the ultrastructural level suggest that sperm morphology relates to the mode of fertilization (internal versus external) [8, 10, 24] and is of importance in phylogenetic reconstruction [7, 10, 11]. Furthermore, sperm morphology assists in distinguishing between families [25] and even between genera [6] and closely related species [4, 9, 20, 23].

In their work on the *Hyla rubra* group, FOUQUETTE & DELAHOUSSAYE [7] regarded sperm structure as important in phylogenetic reconstruction, rather than an adaptation to the fertilization environment. LEE & JAMIESON [12] used phylogeny as an important basis for their work on the ultrastructure of myobatrachid frog sperm. In a later work, JAMIESON *et al.* [10] compared anuran and urodele sperm to that of *Ascaphus*. In this study they indicated which ultrastructural features of sperm could be considered primitive (plesiomorphic), associated with distinct advanced characteristics (autopomorphies), and derived features which were considered paedomorphic. It should be realized that sperm of an individual species may exhibit combinations of these features and that this may complicate interpretation of their phylogenetic position. In addition the latter authors [10] also indicated the relationship of these features to the fertilization biology of amphibians and suggested that the general trend towards simplification of sperm in anurans is a result of secondary reversion to external fertilization.

Among vertebrates, amphibians represent the widest range of "fertilization environments". Internal fertilization is evident in most salamanders and in all caecilians. In anurans such as *Ascaphus* internal fertilization takes place and their sperm are classified as introsperm by JAMIESON & ROUSE [11] and modified sperm by FRANZÉN [8]. External fertilization is the rule for most anurans and their sperm can be classified as ect-aquasperm [11] or in the terminology of FRANZÉN [8] as primitive. The terminology "primitive" used by FRANZÉN [8] is, however, confusing in terms of anurans since they are mainly external fertilizers but have modified sperm. Furthermore, the mode of external fertilization varies among anurans. Direct sperm deposition on the eggs occurs in *Arthroleptella lightfooti* and *Breviceps gibbosus* and sperm only swim through the mucous/egg jelly surrounding of the egg but are not substantially in contact with external fluids or fluids that are not largely from a biological origin of the species themselves. Species exhibiting this pattern will here be referred to as terrestrial fertilizers (TF). In *Bufo* spp. and *Xenopus laevis*, however, sperm are ejaculated in fresh water and sperm actually swim through an external medium to reach the eggs. Species exhibiting this pattern will be referred to as aquatic fertilizers (AF) [24]. It is therefore evident that amphibians represent an ideal model to investigate the relationship of sperm structure to their different fertilization environments.

It can furthermore be expected that sperm shape (particularly the head) and flagellar beat will largely determine the swimming behaviour of sperm. The importance of sperm motility is that it represents a summation of form, mitochondrial function, membrane integrity, exchange of important ions such as calcium and unmasking of receptors. It may be possible that specific sperm motility patterns are associated with mode of fertilization and also with species specific motility patterns.

Our aims in this investigation are to compare sperm structure in the urodeles, caecilians and anurans with special reference to seven of the nine South African anuran families. Here fertilization biology is contrasted with features that are important in establishing phylogenetic relationships. Furthermore, we investigate quantitatively sperm motility patterns in anurans as a function of their fertilization biology. In this part of the investigation we study quantitative sperm motility in representative examples of frogs exhibiting either the terrestrial mode of fertilization or the aquatic mode of fertilization. Lastly, we ascertain whether sperm motion is species specific in anurans.

Source : MNHN, Paris

MATERIALS AND METHODS

General. All comparisons that relate to sperm of the urodeles are extracted from the literature (Table 1). Sperm of only six caecilians have been described in the literature [16, 17, 18, 22], including only one paper on ultrastructure, that of *Typhlonectes natans* [22]. The data on anuran sperm in this paper refer to our original research and representatives of seven of the nine South African families are included. Table 1 refers to lissamphibian species used for comparisons in this investigation.

Capture of animals All anurans used in this study were captured at night during the peak of their breeding season. Animals were transported to the laboratory, were anaesthetized with MS222, dissected and sperm aspirated from the testes within 48 hours of capturing.

Sperm aspiration Testes were removed and all superficial blood vessels and connective tissue were removed. One testis of each specimen was put between two glass slides and squashed. Mainly intact sperm and testicular fluid could be aspirated by means of a micro-pipette. Five µl of this sperm suspension was used for scanning electron microscopy, 5 µl for motility studies and 5 µl for making sperm smears. The other testis was cut into smaller blocks and prepared for transmission electron microscopy.

SEM and TEM preparation Sperm suspension obtained as indicated above was processed for scanning electron microscopy according to the method of VAN DER HORST *et al.* [21]. Small testes blocks were fixed in Sørenson's phosphate buffered glutaraldehyde (2,5%) and post-fixed in 1% osmium tetroxide in the same buffer. Routine processing followed and the material was embedded in Spurr's resin.

Sperm dimensions Sperm were incubated in a nigrosin-eosin solution, sperm smears made and used for measurement of sperm dimensions (acrosome length, sperm head length, sperm head width) using a Kontron image analyzer.

Sperm motility Distilled water, pond water and various physiological media were used at different concentrations (0-300 mOsm/Kg) to establish the ideal medium for activating anuran sperm. HAM's F10 culture medium at 30 mOsm/kg provided the best medium and also preserved motility in most species for several hours [24]. Five ml of sperm suspension was placed in a microscopic bath containing 1 ml of HAM's F10 medium and sperm motion was recorded by means of a video-camera. The images were later replayed and detailed sperm motion characteristics analysed in the fully automated mode by means of computer aided sperm motility analysis (CASMA) utilizing the Sperm Motility Quantifier (SMQ, Wirsam Scientific, South Africa). The curvilinear velocity (VCL), the straight line velocity (VSL), the linearity (LIN), the average path velocity (VAP), amplitude of lateral head displacement (ALH) and dance (DNC = VCL x ALH) were measured. Fig. 1 diagrammatically depicts a typical sperm swimming trajectory and shows the applicable terminology.

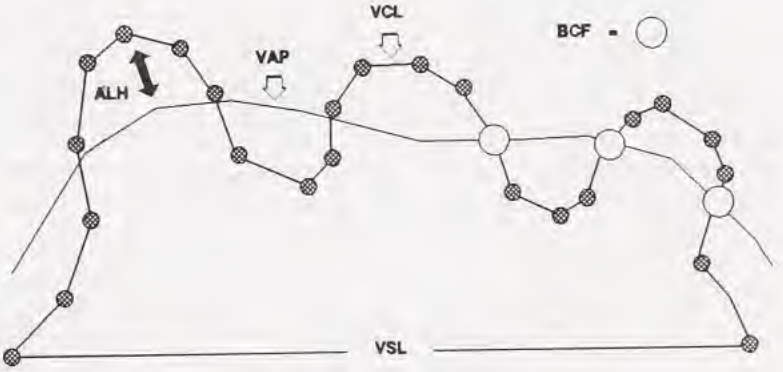


FIG. 1. — Sperm track indicating relevant terminology. VCL, curvilinear velocity; VSL, Straight line velocity; ALH, Amplitude of lateral head displacement; BCF, Beat cross frequency. LIN = Linearity = VSL/VCL.

Source : MNHN, Paris

TABLE 1. — List of species used for drawing comparisons among the Lissamphibia. Only South African anurans which represent our original research are listed. (TF) = Terrestrial fertilizers and (AF) = aquatic fertilizers. Other Anurans are used for comparison in text.

Order	Family	Species	Reference
Urodela	Plethodontidae	*Plethodon albagula*	[10]
	Amphiumidae	*Amphiuma tridactylum*	[2]
	Salamandridae	*Triturus viridescens*	[3]
		Pleurodeles waltlii	[14]
	Proteidae	*Necturus maculosus*	[1]
	Cryptobranchidae	*Cryptobranchus alleganiensis*	[1]
	Ambystomidae	*Ambystoma maculatum*	[3]
Gymnophiona	Ichthyopiidae	*Ichthyopis glutinosus*	[16, 17]
		Uraeotyphlus narayani	[18]
	Caeciliidae	*Siphonops annulatus*	[16]
		Gegeneophis carnosus	[18]
	Typhlonectidae	*Typhlonectes natans*	[22]
		Chthonerpton indistinctum	[5]
Anura	Bufonidae	*Bufo rangeri* (AF)	[24, Present study]
	Hemisotidae	*Hemisus marmoratus* (TF)	[24, Present study]
	Hyperoliidae	*Hyperolius horstocki* (AF)	[24, Present study]
		Semnodactylus wealii (AF)	[24, Present study]
		Leptopelis flavomaculatus (TF)	[24, Present study]
	Mycrohylidae	*Breviceps gibbosus* (TF)	[24, Present study]
	Pipidae	*Xenopus laevis* (AF)	[19, 24, Present study]
	Ranidae	*Arthroleptella lightfooti* (TF)	[24, Present study]
		Pyxicephalus adspersus (AF)	[24, Present study]
		Rana fuscigula (AF)	[24, Present study]
		Strongylopus grayii (AF)	[24, Present study]
		Tomopterna delalandii (AF)	[24, Present study]
	Rhacophoridae	*Chiromantis xerampelina* (TF)	[24, Present study]

Source : MNHN, Paris

RESULTS

Structural considerations of South African anurans

Figs. 2 to 8 are scanning and transmission electron micrographs which represent the full spectrum of sperm morphology in investigated South African anurans and Table 2 represents detailed measurements of these sperm. Representative examples of anurans which exhibit the aquatic mode of fertilization will first be described.

The simplest sperm is that of *Rana fuscigula* which has a straight, short symmetrical head, a short midpiece and a single flagellum (Fig. 2). The acrosomal cap is short. The sperm of *Semnodactylus wealii* have similar features except that the flagellum has in addition an undulating membrane and axial rod (Fig. 3). *Xenopus laevis* has a single flagellum but the head is partly corkscrew shaped with one and a half coils (Fig. 4). *Xenopus laevis* sperm furthermore differ from most anurans in that the first few rows of mitochondria surround the posterior part of the nucleus (Fig. 4, insert). *Bufo rangeri* sperm have spear-shaped heads, distinct but short midpieces and flagella with undulating membranes and axial rods (Fig. 5). The head length of aquatic fertilizers ranges from 10.3 to 20.5 µm.

In contrast the sperm of South African anurans which exhibit the terrestrial mode of fertilization have very long and slender heads (*Hemisus marmoratus*, *Leptopelis flavomaculatus*, *Breviceps gibbosus*, *Arthroleptella lightfooti*) or long heavily coiled heads (*Chiromantis xerampelina*) varying from 21.4 to 45 µm in length (Figs 6 & 8) (Table 2). Both the head lengths as well as the acrosome lengths of the terrestrial fertilizers were significantly ($p<0.05$) longer than those of the aquatic fertilizers. However, the flagella of the TF represent either a single axoneme or an axoneme with an undulating membrane and axial rod like the AF. The exception was *Chiromantis xerampelina* which has a single flagellar complex containing two axonemes surrounded by a multitude of microtubules. The term ect-terrasperm is suggested as a new terminology for amphibians which exhibit the terrestrial mode of fertilization. A clear distinction between ect-aquasperm and ect-terrasperm is therefore evident.

In *Chiromantis* the first few rows of mitochondria also surround the posterior part of the nucleus as in *Xenopus*. Furthermore, *Chiromantis* sperm has spherical mitochondria (Fig. 7) in contrast to *Rhacophorus arboreus* sperm which has elongate mitochondria.

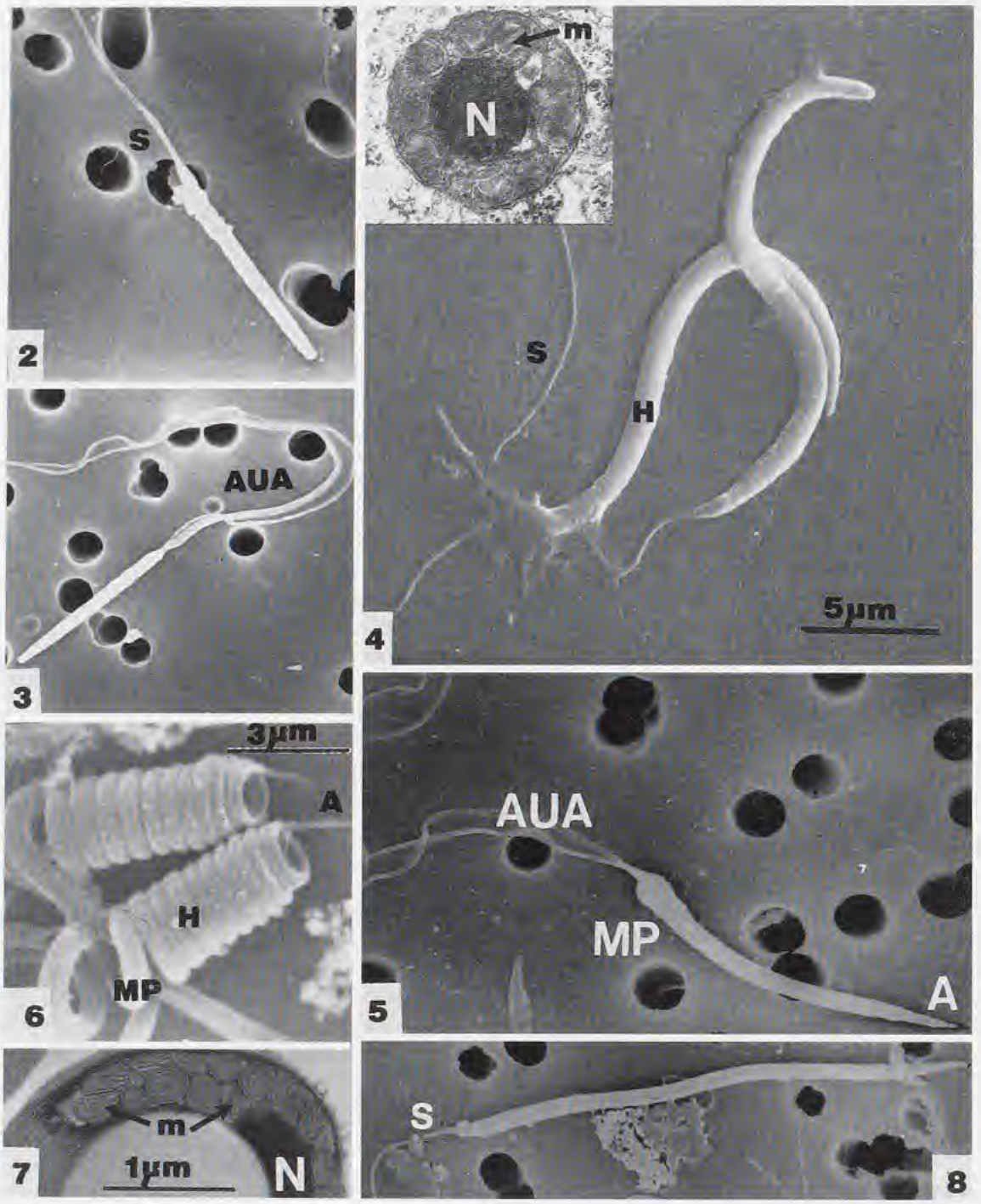
Urodeles and caecilians

Sperm structure in the urodeles and caecilians (limited information) shows less variation than the anurans in terms of basic design. The sperm heads in both these groups are long and slender. While considerable variation exists in the size and form of the flagellum, the basic pattern of axoneme-undulating membrane-axial rod (AUA) seems to occur in all urodele and caecilian species. Their acrosomes are similar in that they extend from the anterior part of the nucleus but do not form a cap that largely overlaps with the nucleus as in anurans. The detailed structure of the acrosome of urodeles and caecilians are also complex and mostly contain an acrosomal rod surrounded by less dense but granular material. All urodele sperm furthermore contain a marginal filament or juxta-axonemal fibre at doublet 8 next to the axoneme, which is absent in caecilians and anurans.

Sperm motility of South African anurans

Sperm of all aquatic fertilizers exhibited forward progression and the VCL varied from 20 to 31 µm/s, VSL from 8 to 23 µm/s, LIN from 40 to 72%, BCF from 3 to 6Hz and ALH from 4.7 to 7.5µm. In contrast the sperm of two species of terrestrial fertilizers (*Breviceps* and *Arthroleptella*) were immotile in a wide range of physiological media and osmotic concentrations varying from 10 to 300 mOsm/kg. In *Chiromantis xerampelina*, sperm only exhibited an initial rapid starspin movement followed by uncoiling of the head and became immotile within a

Source : MNHN, Paris



FIGS 2-8. — Scanning electron micrographs of amphibian spermatozoa. **2:** *Rana fuscigula*. **3:** *Semnodactylus wealii*. **4:** *Xenopus laevis* (inset: transmission electron micrograph of midpiece). **5:** *Bufo rangeri*. **6:** *Chiromantis xerampelina*. **7:** *Chiromantis xerampelina*, transmission electron micrograph of midpiece. **8:** *Breviceps gibbosus*. A, acrosome; AUA, Axoneme-undulating membrane-axial rod; H, Head; m, mitochondria; MP, midpiece; N, Nucleus. S, single flagellum only with axoneme. Holes in membrane filters (Figs 2, 3, 5 & 8) are 3 µm in diameter.

Source : MNHN, Paris

minute. Star symbol plots were used to visually express sperm motility as a pattern of movement (Fig. 9). Distinct motility patterns could be constructed that were clearly species specific. Sperm with partly coiled heads such as *Xenopus* and *Strongylopus* swim in a corkscrew fashion and the linearity of the sperm is almost similar (41.3% and 42.4% respectively) whereas *Bufo* sperm have straight heads and a LIN of 67.6%. Furthermore, more closely related species (*S. grayii* and *P. adspersus*) had distinct sperm motility patterns but also shared many similarities for several motion parameters. This can also be seen in Fig. 9, indicating the similarity in star symbol plots.

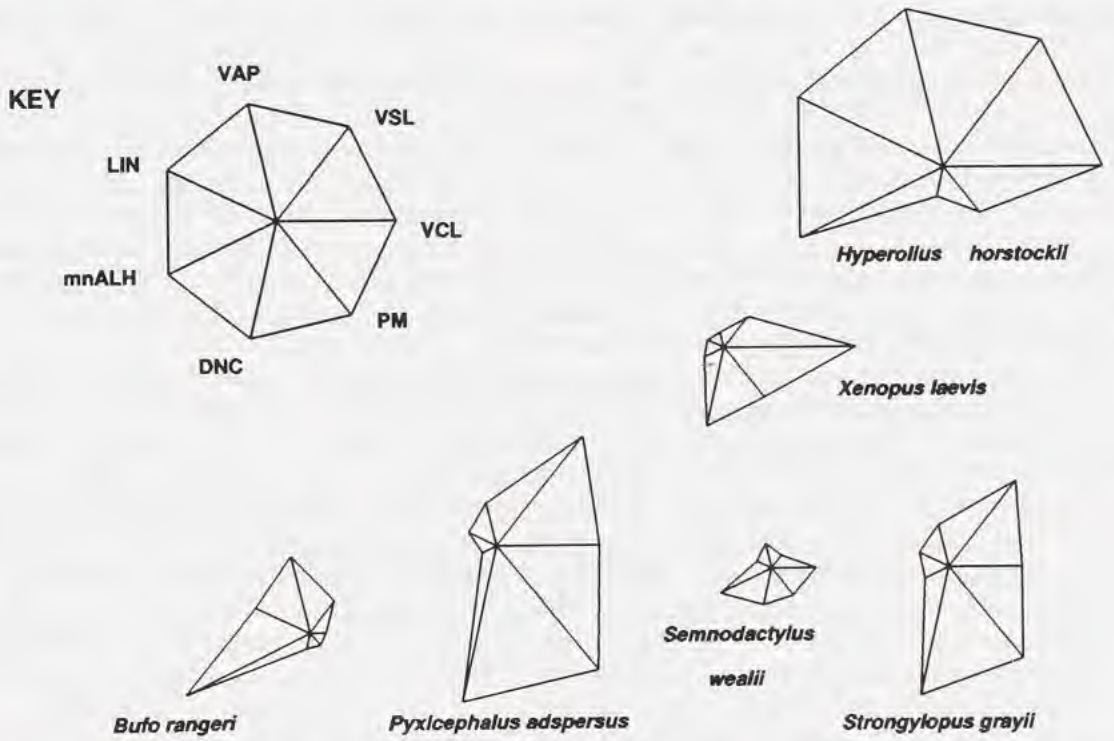


FIG. 9. — Star Symbol Plots (seven motion parameters used - see abbreviations elsewhere in text - PM = percent motile sperm) depicting patterns of sperm motion among six anuran species. For any set of stars (above) the smallest observed value for a parameter is plotted as an arm one-tenth the length of the arm representing the largest observed value.

TABLE 2. — Sperm dimensions of aquatic and terrestrial South African anuran fertilizers.

Fertilization grouping	Head length (µm)	Head width (µm)	Acrosome length (µm)
Aquatic			
mean	16.0	0.7	2.2
(± SD)	(2.2)	(0.4)	(1.2)
Range	10.3 - 20.5	0.1 - 1.6	0.7 - 4.6
Terrestrial			
mean	29.6	0.6	5.9
(± SD)	(6.0)	(0.3)	(1.6)
Range	21.4 - 45.0	0.1 - 1.1	3.2 - 7.3

Source : MNHN, Paris

DISCUSSION

It has been hypothesized that primitive lissamphibians were internal fertilizing and that external fertilization was a secondary reversion in the anurans [10]. The 9+2 axoneme-undulating membrane-axial rod is typically associated with this pattern as well as distinct acrosomal and neck piece features. In reptiles, birds and mammals, where internal fertilization is the rule, a flagellum is associated with a 9+9+2 pattern. There, the nine outer coarse fibres seem to function as a strengthening device for sperm swimming in a viscous environment. The juxta-axonemal fibre as well as the axial rod in amphibians seem to be homologous to the coarse outer fibres 8 and 3 of amniotes and insects [14]. One interpretation may be that the AUA of the early internal fertilizing amphibians represent an intermediate structure from external to internal fertilization and this has been retained as a primitive feature in reversion to external fertilization in many anurans. Alternatively, the AUA of early internal fertilizing Lissamphibia may already represent a simplification of an ancestor that had a 9+9+2 flagellar arrangement. Our data agrees with the interpretation that this feature (AUA) in anurans reflects a plesiomorphic character within a particular group or family [7, 10]. A comparison of three species of *Heleophryne* furthermore supports this view [23]. The flagellar arrangement of particularly the Anura is therefore one of the most important features in determining the relative phylogenetic position of a species. Our results confirm a change from the AUA to a flagellum without an axial rod when examining the more primitive to the more advanced South African anurans.

Our results furthermore show a distinct relationship between sperm head length and mode of fertilization among external fertilizing anurans. The sperm heads and acrosomes of aquatic fertilizing anurans are significantly shorter than those of the TF. It is also known that the egg coverings of TF are generally thicker than those of AF, presumably to protect the eggs against dehydration [13]. A longer sperm head and a larger acrosome which may contain more digestive enzymes may have been an advantage in penetrating these thick egg coats [24]. The information on head and acrosome length may also be of predictive value in other anuran species in establishing whether they belong to the TF or AF grouping. Three South African *Heleophryne* species posses sperm heads with lengths varying from 23 to 28 μm [23]. They are predominantly TF and further support our results. It is therefore clear from our data on South African anurans that sperm head and acrosome dimensions reflect on the fertilization biology rather than the phylogeny and is independent of the type of flagellum (only axoneme or with undulating membrane and axial rod).

The sperm of the three orders of Lissamphibia can be distinguished on the basis of distinct sperm features. A diagnostic feature for urodele and caecilian sperm is a complex acrosome that predominantly extends from the anterior part of the head and does not form a cap surrounding the anterior tip of the nucleus as in all anurans and an acrosomal cap is therefore a diagnostic feature for anurans. Here *Ascaphus* [10] seems to occupy an intermediate position in having a complex acrosome like the urodeles and caecilians but it also forms a cap around the anterior tip of the nucleus. A juxta-axonemal fibre at position 8 is a diagnostic feature for urodeles and separates them from the caecilians and anurans. Anurans on the other hand have developed a minor juxta-axonemal fibre at position 3 which is not clearly defined in urodeles and apparently absent in caecilian sperm [22]. Glycogen packets have furthermore been observed in two ranid species [15] and in *Xenopus* [19] and while the occurrence of these packets is uncommon, they seem to be specific for anurans. *Chiromantis* sperm have spherical mitochondria in contrast to *Rhacophorus* sperm which have elongate mitochondria. The spherical mitochondrion appears to represent the primitive condition in anurans [10] and this may suggest a plesiomorphic character for *Chiromantis* in relation to *Rhacophorus*.

Further sperm structural features that seem to reflect on the fertilization biology of urodeles and caecilians will be briefly discussed. A correlation appears to exist between the length of the neck-piece and the length of time that the sperm is retained in the female cloaca. In

Source : MNHN, Paris

Cryptobranchus external fertilization is the rule and the neck-piece is very small whereas in *Diemictylus*, where sperm are retained in the cloaca for several months, the neck-piece is long [3]. In two species of viviparous Typhlonectidae the acrosome is barbed or hooked [6, 22]. However, in four oviparous taxa of caecilians the acrosomes are spatulate [16, 17, 18]. It appears that the shape of the acrosome of caecilians may be predictive in determining oviparity or viviparity of a given species [22].

Our results on quantitative sperm motility in anurans indicate that sperm from all AF possess motile sperm that swim progressively forward in a physiological medium of 30 mOsm/kg. In contrast sperm from TF are immotile in a wide range of physiological and culture media ranging from 10 to 300 mOsm/kg. In AF, sperm swim in a low osmolality environment even if the male and female cloacae are in close proximity such as in *Xenopus*. Sperm of *Chiromantis xerampelina* (TF) only exhibited a brief spurt (seconds) of hyperactivated motility. In TF the sperm are deposited directly on the eggs and the need for vigorous and longer term motility seems less than in the AF group. It should also be conceded that sperm activation in TF may be dependant on specific substances associated with the egg coat/surface and may explain the immotile status of TF sperm in our experiments.

Finally sperm motion is highly species specific among the South African anurans. The pattern of sperm motion appears to be related to the form of the sperm head, the type of tail (presence or absence of undulating membrane) and the type of flagellar beat. The AF anurans with only an axoneme seem to exhibit greater values for most motion parameters than those with an axoneme-undulating membrane-axial rod and the AF accordingly have larger star symbol plots (Fig. 9).

In summary it appears that several sperm structural features in the Lissamphibia are diagnostic in separating the three main orders, families (urodeles), and even closely related species (*Heleophryne*). It furthermore assists in phylogenetic inferences and is predictive in terms of fertilization biology. Quantitative sperm motility analysis of representative examples of seven of the nine South African anuran families suggest that sperm motility is species specific and also relates to fertilization biology.

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Source : MNHN, Paris

Evolution of Tetrapod Spermatozoa with Particular Reference to Amniotes

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ABSTRACT

Synapomorphies of tetrapod sperm appear to be: nuclear 'shoulders'; elongation, relative to dipnoans, of two longitudinal elements (dense fibres) peripheral to the axoneme adjacent to doublets 3 and 8; and, questionably, development of an annulus. A lissamphibian synapomorphy relative to *Neoceratodus* may have been loss of one undulating membrane, leaving a single undulating membrane adjacent to the fibre of doublet 3. Amniote synapomorphies (retained in Chelonia and Sphenodontida) include: elongation of the distal centriole through the entire length of the moderately elongate midpiece; subspheroidal mitochondria, with concentric cristae; a fibrous sheath; nine peripheral axonemal fibres; inward projections (longitudinal columns) of the fibrous sheath aligned with fibres 3 and 8; loss or transformation of the retronuclear body, present in dipnoans and (as the neck structure) urodeles. A possible crocodilian synapomorphy is a thick dense sheath around the singlets of the axoneme or the distal centriole. Synapomorphies of birds are loss of the subacrosomal cone and, less certainly derived, adhesion of all nine dense fibres to their axonemal doublets (also in monotremes). The conical acrosome, fibrous sheath, and elongate centriole of ratites are symplesiomorphies not proving monophyly. Restriction of the endonuclear canal to the anterior region of the nucleus in other non-passerines and passerines may be a synapomorphy of these, homoplasic with crocodiles and derived ratites (emu). Squamate synapomorphies are: loss of endonuclear canals with restriction of the perforatorial rod to a prenuclear location; intermitochondrial bodies; forward extension of the fibrous sheath into the midpiece; a paracrystalline subacrosomal cone; and, homoplasically, shortening of the centriole. Mammal sperm are distinguished by loss of the perforatorium (and canal), homoplasic with some non-ratite birds, great reduction of the centrioles, and, in therians, (apomorphic?) detachment of peripheral fibres, except sometimes 3 and 8, from the doublets.

RÉSUMÉ

Évolution des spermatozoïdes des Tétrapodes, en particulier des Amniotes

Les synapomorphies des spermatozoïdes des tétrapodes semblent être: des "épaules' nucléaires; l'élongation, en comparaison des Dipneustes, de deux éléments longitudinaux (fibres denses) en périphérie de l'axonème et adjacents aux doublets 3 et 8; et de manière incertaine, le développement de l'annulus. Une synapomorphie des Lissamphibiens en comparaison de *Neoceratodus* peut être la perte d'une membrane ondulante, ce qui laisse une seule membrane ondulante adjacente à la fibre du doublet 3. Les synapomorphies des Amniotes (conservées chez les Chelonia et les Sphenodontia) comprennent: l'élongation du centriole distal sur toute la longueur de la pièce intermédiaire, qui est modérément allongée; des mitochondries subsphériques, avec des crêtes concentriques; une gaine fibreuse; neuf fibres axonémales périphériques; des projections vers l'intérieur (colonnes longitudinales) de la gaine fibreuse alignées avec les fibres 3 et 8; la perte ou la transformation du corps rétronucléaire, présent chez les Dipneustes et (comme structure du cou) chez les Urodèles. Une synapomorphie possible des Crocodiliens est la gaine dense épaisse entourant les singulets de l'axonème ou le centriole distal. Les synapomorphies des Oiseaux sont la perte du cône subacrosomien et l'adhésion des neuf fibres denses à leurs

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Source : MNHN, Paris

doublets respectifs. Cette dernière structure existe aussi chez les Monotrèmes et son caractère évolué est moins assuré. L'acrosome conique, la gaine fibreuse et le centriole allongé des Ratites sont des symplésiomorphies qui ne prouvent pas leur monophylie. La restriction du canal endonucléaire à la région antérieure du noyau chez les autres non-passereaux et chez les Passereaux peut être une synapomorphie pour ces groupes, homoplasique avec les Crocodiles et les ratites évolués (Ému). Les synapomorphies des Squamates sont: la perte des canaux endonucléaires avec la restriction de la baguette du perforatorium à une position pré-nuclaire; des corps intermitochondriaux; une extension vers l'avant de la gaine fibreuse dans la pièce intermédiaire; un cône subacrosomien paracristallin; et de manière homoplasique, le raccourcissement du centriole. Les spermatozoïdes des Mammifères se distinguent par la perte du perforatorium (et du canal) qui est homoplasique avec certain Oiseaux non-ratites, la grande réduction des centrioles, et, chez les Thériens (apomorphie?), le détachement des fibres périphériques, exceptées parfois les 3 et 8, des doublets.

This chapter constitutes a brief review, with new material, of the sperm of the amniotes as a whole and attempts to reconstruct the features of the spermatozoon of the ancestral Amniota and the course of spermatozoal evolution, as indicated by deduced synapomorphies, in the amniote classes. This reconstruction is based on intuitive consideration, and also on a cladistic analysis [22], of spermatozoal ultrastructure in the constituent tetrapod groups. Further reviews of the ultrastructure of vertebrate sperm may be found in the recent publications of JAMIESON [21] and HEALY & JAMIESON [16, 22]. Material of dipnoan and urodele sperm is included for comparative purposes.

MATERIALS AND METHODS

Testes and ducts were dissected from euthanased specimen(s) of *Neoceratodus forsteri* (Dipnoi), *Taricha granulosa* (Urodela), *Emydura kreffti* (Chelonia), *Sphenodon punctatus* (Sphenodontida), *Crocodylus johnstoni* (Crocodilia), *Lampropholis delicata* (Scincidae, Squamata), *Geopelia striata* (Columbiformes, Aves) and *Rhinolophus megaphylla* (Chiroptera, Mammalia). Processing of the tissues was as in [27].

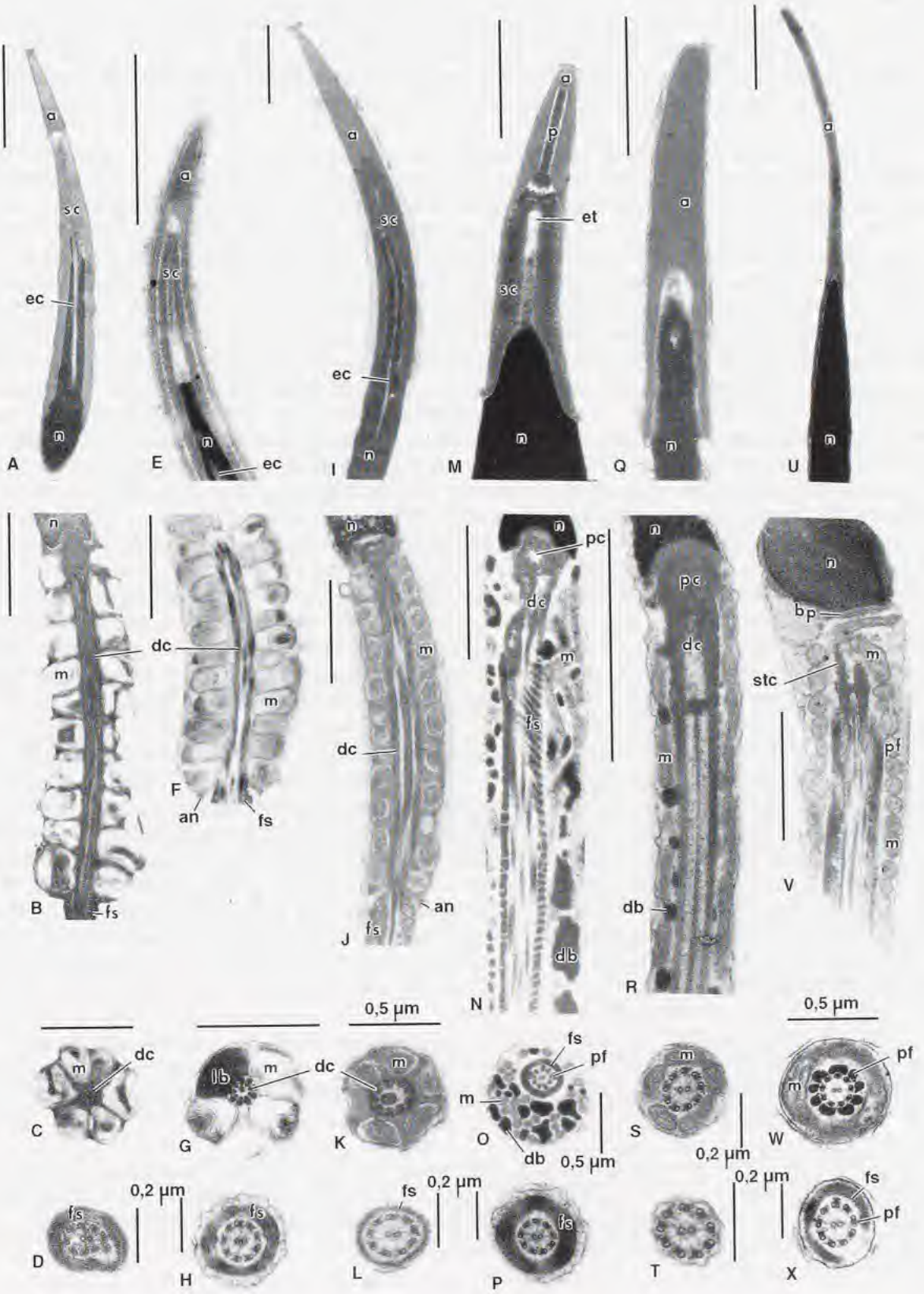
RESULTS AND DISCUSSION

Comparative ultrastructure of amniote spermatozoa

The acrosome, endonuclear canals and perforatoria. All amniote classes (Reptilia, Birds and Mammals) contain some species, or a majority of species, in which the acrosome has a plesiomorphic tripartite structure which, from the evidence of its presence in all three lissamphibian orders (Urodela, Gymnophiona and Anura) [25], was already present in early tetrapods ancestral to Lissamphibia and Amniota. In the plesiomorphic tripartite acrosome the acrosome vesicle forms an elongate, narrow cone symmetrically located on the tip of the nucleus which narrows within it to a point. The acrosome vesicle encloses a similarly shaped subacrosomal cone and axially within this there are one to three rodlike perforatoria. The pointed form of the acrosome, presence of the subacrosomal cone, and tapering of the tip (rostrum) of a

FIG. 1. — Examples of sperm ultrastructure in selected amniote classes and orders. The species depicted in each vertical column are **1:** *Emydura kreffti* (Chelonia); **2:** *Sphenodon punctatus* (Sphenodontida); **3:** *Crocodylus johnstoni* (Crocodilia); **4:** *Lampropholis delicata* (Scincidae, Squamata). **5:** *Geopelia striata* (Columbiformes, Aves); and **6:** *Rhinolophus megaphylla* (Chiroptera, Mammalia). The first transverse row, A, E, I, M, Q, and U, are longitudinal sections through the sperm head (acrosome and anterior nucleus). The second row, B, F, J, N, R, and V, are longitudinal sections through the midpiece and, in all except F, the posterior region of the nucleus. The third row, C, G, K, O, S, W, are transverse sections through the midpiece. The fourth row, D, H, L, P, T and X, are transverse sections through the principal piece. Abbreviations: a, acrosome vesicle; an, annulus; bp, basal plate; db, dense bodies (mitochondrial transformations); dc, distal centriole; ec, endonuclear canal; et, electron-lucent epinuclear region; fs, fibrous sheath; lb, lateral body at anterior region of distal centriole; m, mitochondria; n, nucleus; p, putative perforatorium; pc, proximal centriole; pf, peripheral dense (coarse) fibres; sc, subacrosomal cone; stc, striated column. Scale bar = 1 µm unless otherwise indicated.

Source : MNHN, Paris



Source : MNHN, Paris

cylindroid nucleus within this, are seen in the Chelonia [13, 16, 19, 22] (Fig. 1A), Crocodilia (*Caiman crocodylus* [45], and *Crocodylus johnstoni*, Fig. 1I), Sphenodontida (*Sphenodon*) [16, 22] (Fig. 1E), Squamata [5, 6, 13, 27] (Fig. 1M), non-passerine birds [24, 37] (Fig. 1Q), and monotremes [7]. All of these plesiomorphic representatives of their classes, with the exception of mammals, and, in the non-passerines, the columbiforms (Fig. 1Q), possess the perforatorial rod or rods.

In the Squamata the subacrosomal cone has a paracrystalline substructure [5, 6, 13], recently confirmed for Sphenomorphus and Eugongylus group skinks, the gekkonid *Heteronotia binoei*, and in snakes [35]. It constitutes a basal synapomorphy of the Squamata [22].

The perforatorial rod in the Squamata is wholly prenuclear (Fig. 1M) but this restriction is clearly apomorphic. The plesiomorphic condition is seen in basal lissamphibians where a rod penetrates the nucleus to varying depths, each within an endonuclear canal: *viz.* urodeles [41] and the primitive anurans *Ascaphus* [25], *Discoglossus* (spermatid only) [47, 48], and bombinids, including *Bombina* and *Alytes* [43]. The number of endonuclear canals and of enclosed perforatoria is one in basal Lissamphibia, in the caiman (though poorly substantiated by micrographs), tinamou, rhea and non-passerines (e.g. galliforms), but in the Chelonia and *Crocodylus johnstoni* there are two or three canals and there are two in *Sphenodon*. There are three endonuclear canals in the sperm of the sturgeon, *Acipenser sturio*. Although there appears to be only a single canal in the coelacanth, *Latimeria chalumnae*, this contains two or three perforatoria [21] and two to four perforatoria are seen in *Neoceratodus* (Fig. 3B). It is therefore probable that the presumed common ancestor of Amphibia and amniotes possessed more than one perforatorium and endonuclear canal. A single canal appears basic to all amniotes above turtles and *Sphenodon* [22]. In *Acipenser* the canals are spiralled around each other as they are in turtles, *Sphenodon* and *Crocodylus johnstoni*. The spiral arrangement or at least the presence of one or more endonuclear canals may well be a synapomorphy for the Osteichythes, a monophyletic clade including the Actinopterygii (Ray-finned fish), Sarcopterygii and, within the latter, the Tetrapoda. That a canal was present even earlier is evidenced by presence of a canal and filamentous perforatorium in lamprey sperm. The canals are absent (presumed lost) in the highly simplified sperm of the Chondrostei and Neopterygii [21].

In birds, a conical acrosome vesicle penetrated almost to its tip by a subacrosomal space which contains a rodlike perforatorium has been demonstrated ultrastructurally in the non-passerines turkey, *Meleagris gallopavo*, chicken, *Gallus domesticus*, guinea fowl, *Numida meleagris* (e.g. [55]), the mallard duck, *Anas platyrhynchos* [20], and the quail *Coturnix coturnix* [20], parrots [24] and in the ratites (palaeognaths) tinamou, *Eudromia elegans* [1], ostrich, *Struthio camelus* [3, 51, 52] and emu, *Dromaius novaehollandiae* [3]. Like the sperm of ratites and other birds, parrot sperm differ from those of reptiles in reduction of the subacrosomal material (subacrosomal cone, excluding any perforatorium) to a negligible amount [24]. In the columbiforms even a perforatorium is absent although, at least in *Geopelia striata* (Fig. 1Q), some longitudinally orientated subacrosomal material is present and lacunae are present in the nucleus which may represent a vestigial endonuclear canal.

Not all non-passerines possess a conical acrosome. A small, approximately spherical acrosome has been described for the white-naped crane, *Grus vipio* [40], for *Jacana jacana* [46] and most Charadriiformes [11], and for the wood pecker *Melanerpes carolinus* ([18]. These latter avian taxa are considered to be advanced non-passerines, on the basis of DNA hybridization studies [49, 50].

In the non-passerine and suboscine spermatozoon the acrosome is short relative to the nucleus, as in reptiles [22, 27, 28], in contrast to the oscine spermatozoon which has an extremely large acrosomal complex. In passerines the acrosome vesicle becomes an elongate single-keeled helix, with no evident subacrosomal cone, in, for instance, finches [30, and KOEHLER, this volume].

Source : MNHN, Paris

The endonuclear canal extends almost to the base of the nucleus in Chelonia and *Sphenodon* [16, 17, 22], and this is considered a plesiomorphic state [22]. This condition also occurs in putatively more primitive ratites (i.e. tinamou [1]), where it is probably also plesiomorphic relative to the shorter condition in other non-passerines and in advanced ratites, being wholly prenuclear in the emu [3]. However, there is a possibility that deep penetration in ratites is secondary [22]. The canals extend deeply into the nucleus in *Crocodylus johnstoni* (Fig. 1I). A single canal is depicted as penetrating only the nuclear rostrum in the caiman [45] but the extent requires further investigation. The endonuclear canal is limited to the anterior 1 to 2 μm of the nucleus in rooster, guinea fowl, turkey and parrots and the anterior third of the nucleus in the ostrich [3]. The canal is lost in mammals and, homoplasically, in squamates.

In mammals, as in the bat *Rhinolophus megaphylla* (Fig. 1U), rodlike perforatoria do not occur. Although monotremes [7], retain the elongate conical structure of the acrosome and the subacrosomal cone, this form is greatly modified in the Eutheria (e.g. [44]) and Metatheria [53]. In Eutheria the acrosome is flattened, with the notable exception of the pangolin [33].

The nucleus and nuclear fossa. The nucleus is plesiomorphically elongate in amniotes from Chelonia through *Sphenodon*, crocodiles, squamates, birds, monotremes and, in therian mammals, the pangolin alone [33], as in lissamphibians.

Representation of the basal nuclear fossa, loosely termed the implantation fossa, is variable in amniotes but some of this variation may be spurious as it is difficult to establish the shape of the fossa. It appears poorly developed in the sperm of *Caiman crocodylus* [45] but has a low dome-shaped form in *Crocodylus johnstoni* (Fig. 1J). It is small and compact in turtles, tuatara, rooster, guinea fowl, and squamates excepting some skinks in which it is narrowly funnel-shaped. In the ratites it has a a triple profile (references in [22]). It is dome-shaped to rounded conical in eugongyloid skinks [28]. It is a shallow cone in the gekkonid *Heteronotia binoei* and compactly conical in the pygopodid *Lialis burtoni* [26]. The compact form appears to be plesiomorphic for amniotes [22].

The annulus. A dense ring, the annulus, at the posterior end of the midpiece is a feature of many metazoan sperm. It is clearly plesiomorphic for amniotes, occurring in all classes [22] but absence in Dipnoi possibly indicates apomorphic re-acquisition in tetrapods. It is well developed in Chelonia, *Sphenodon* [16, 17, 22], *Caiman crocodylus* [45], the American Alligator [39] and *Crocodylus johnstoni* (Fig. 1J). In squamates, it has been demonstrated for *Lacerta vivipara* [8], *Cnemidophorus sexlineatus* [34], sphenomorph and eugongyloid skinks [27, 28], *Heteronotia binoei*, *Varanus gouldii flavirufus* [35], and, though reduced in some species, in snakes [23, 35]. The annulus is basic to birds, being seen in ratites, rooster, guinea fowl, and columbiforms [2] but is apomorphically absent in parrots [24]. It is weakly developed in monotremes [7]. Two structural categories of annulus have been recognized in therian mammalian sperm, based on the profile of the annulus as viewed in longitudinal section: triangular (e.g. bats, dormouse, Chinese hamster, antelope) and semicircular (e.g. mouse, guinea pig, chinchilla, ram) [12]. However, the taxonomic and phylogenetic significance of the shape of the annulus is questionable, given that in the Rodentia both categories are encountered.

The number of mitochondria. The number of mitochondria seen in transverse section of the midpiece is very variable in amniotes but some of the apparent variation requires confirmation, particularly as there is variation along the midpiece. Determination of the total number by scanning electron microscopy would be desirable. It appears that a number, in transverse section, in the order of 6 to 9 may have been plesiomorphic; 6 has been recorded for the Chelonia, 9 in *Sphenodon*, 6 to 8 in *Caiman crocodylus* [22, 45] and 6 in *Crocodylus johnstoni* (Fig. 1K). In the remaining amniotes, a trend towards reduction, in transverse section, to 4 in birds and monotremes has been suggested [22]. It is 4 in ratites (e.g. [51]) and in the turkey [54] and in the order of 5 in *Geopelia striata* (Fig. 1S). However, in squamates the number remained plesiomorphically high in lizards or showed apomorphic increase to as many as 14, in snakes,

Source : MNHN, Paris

while a reduction to 2 in gekkos was correlated with intrusion of intermitochondrial material of supposed mitochondrial origin into the transverse section of the midpiece. Further variability in numbers is now known though it cannot be fully documented here. For instance, large numbers of small mitochondria occur, it seems apomorphically, in eugongyloid skinks [28] and approximately 9 have been observed in transverse section of the budgerigar sperm [24]. The mitochondria are subspheroidal in Chelonia, *Sphenodon* and crocodiles and this may reasonably be inferred as the plesiomorphic condition. The number of tiers of mitochondria in longitudinal sequence is in the order of 10 in turtles [16] which is also presumed to be plesiomorphic. In the spiral midpiece of mammals, the number of gyres varies from 55 to 300 [10] but is not specified for monotremes [7].

Structure of the mitochondria. In turtles (Fig. 1B, C), tuatara (Fig. 1F, G), *Caiman crocodylus* and *Crocodylus johnstoni* (Fig. 1J, K), the mitochondria have a form known only in the sperm of one other amniote, the Woolly opossum, *Caluromys philander* (see [36]; [9]). The mitochondrial cristae in these three taxa are concentric and usually surround a large central dense body. In all other amniotes studied, the cristae have a "conventional" appearance, being linear or curved, as in Lissamphibia, but never concentric, and do not surround a dense body.

Linear cristae in spermatozoal, as in somatic mitochondria, must be accepted as a plesiomorphic condition for tetrapods as they are normal for metazoan sperm, including fish. The concentric arrangement with dense body appears to be an apomorphy acquired early or initially in amniote evolution and retained paraphyletically in the tuatara, crocodile, and turtle clades [16, 22]. In spermatids of *Caiman crocodylus* [45] and in at least some mitochondria of spermatids of *Sphenodon* [16, 22], the cristae have the linear appearance usual for metazoan sperm and the concentric arrangement is a late development. Phylogenetic "reversion" of mitochondrial of concentric cristae to the linear condition seen in other amniotes would need only suppression of this final transformation [22]. Presence of concentric cristae and the intramitochondrial body in the woolly opossum is construed as homoplasic although the possibility that ancestral mammals retained this condition from basal amniotes cannot be ruled out [16].

The intermitochondrial rings or dense bodies of squamate sperm are regarded as derivations of the intramitochondrial dense bodies [6, 16, 22] and as such a "reminiscence" of the occurrence of concentric cristae in the ancestry of squamates. Origin of intermitochondrial material from mitochondria has been confirmed ontogenetically in the sperm of some squamates [35]. Extramitochondrial dense bodies are almost limited to squamates but are seen, poorly developed, in *Geopelia striata* (Fig. 1R) in which, although appearing homoplasic, they may well indicate persistence of a genetic basis laid down in early amniotes.

The centrioles. Presence of the proximal centriole can be regarded as plesiomorphic for tetrapods and is seen in all amniote classes. It persists, well developed, in monotremes [7], but is absent from mature therian mammals, for instance, the rat [10] and the bat, *Rhinolophus megaphylla* (Fig. 1V).

A distal centriole is at most a vestige in mature mammalian sperm [10] (see also Fig. 1V), but is well developed in sperm of anurans [32], Chelonia (Fig. 1B, C), *Sphenodon* (Fig. 1F, G), crocodiles [16, 22] (Fig. 1J, K), squamates [13] (Fig. 1N), and birds [1, 2] (Fig. 1R). The distal centriole, forming the basal body of the axoneme, is plesiomorphically short in vertebrates, including the Lissamphibia and squamates [27]. In contrast, the distal centriole extends the entire length of the long midpiece in turtles (Fig. 1B, C), the tuatara (Fig. 1F, G), crocodiles (Fig. 1J, K), and ratites, an apparent basal synapomorphy of amniotes. These elongate centrioles differ from most metazoan basal bodies in being penetrated by two central singlets from the axoneme. Thus in spermatids of the ratite *Rhea*, the distal centriole elongates and, late in spermiogenesis, becomes penetrated by a central pair of tubules from the developing axoneme [38]. The shorter, though still elongate distal centriole in the rooster and the somewhat shorter centriole in guinea fowl (0.6 μm) and *Geopelia striata* (0.5 μm) (Fig. 1R), the short centriole in squamates, and the

Source : MNHN, Paris

vestigial centriole in monotremes possibly represent secondary reduction in length of the centriole [16], culminating in almost total reduction in therian mammals.

The distal centriole is embedded in a ring of dense material in all of the amniotes for which it has been investigated. A cross striated dense body lateral to the proximal centriole appears to be a basal synapomorphy of amniotes but its homology across the various groups requires confirmation. It is seen in tuatara, and the caiman where homology with the nine striated columns of eutherian sperm has been suggested [16] but has yet to be demonstrated in *Crocodylus johstoni*. In the skink *Nangura* it is represented by a bilateral laminated structure [27] and shows various manifestations in other squamates. It is does not appear to have been reported in birds.

The axoneme and appurtenances. An annulated, helical, dense fibrous sheath (Fig. 1B, D, F, H, J, L, N-P, X) must, clearly, have developed in the earliest amniotes as it is present in all amniote classes. Homology of this sheath across the different classes is reinforced by the presence in mammals of two spurlike inwardly directed triangular processes, seen in cross section of the sperm, in the vicinity of doublets 3 and 8, where they form large 'longitudinal columns' [10], though at most weakly developed in monotremes, and in ratites. In the non-passerines, rooster and guinea fowl, the fibrous sheath has transformed into an amorphous sheath [55] while in parrots [24] and doves (*Ocyphaps lophotes*, pers. obs., and *Geopelia striata*) it is lost.

In most of the amniotes investigated the fibrous, or amorphous, sheath commences immediately behind the midpiece. This condition is seen in turtles (Fig. 1B), *Caiman crocodylus* and *Crocodylus johnstoni* (Fig. 1J), ratites, non-passerines (absent in parrots and doves) and mammals. However, in squamates the fibrous sheath extends anteriorly well into the midpiece, a clear squamate autapomorphy [16, 22, 27], as in *Lampropholis delicata* (Fig. 1N, O).

An external longitudinal protuberance (rib) on each side of the fibrous sheath is seen in many amniotes, including eutherians, and is particularly well developed in the tinamou [1]. A sheath of putative glycogen external to the fibrous sheath was found to be limited to the tinamou [38] and cannot be ascribed to the plesiomorphic amniote sperm.

Nine longitudinal dense fibres (coarse fibres) peripheral to the nine axonemal doublets, or to the distal centriole also where this is elongated as in *Emydura*, *Sphenodon* and *Crocodylus*, are a fundamental feature of amniote sperm, being found in all classes [22, 27]. The presence of nine fibres is an autapomorphy and simultaneous symplesiomorphy of the amniotes, though nine appear homoplasically in other groups, such as some lampreys and the osteoglossomorph fish *Pantodon* [21], and in heterobranch and cephalopod molluscs [14, 15]. The dense fibres are small in turtles (Fig. 1C), *Caiman crocodylus* and *Crocodylus johnstoni* (Fig. 1K), the tuatara (Fig. 1G), squamates (Fig. 1O), birds and monotremes. They have been observed in turkey, rooster and, though requiring confirmation, in guinea fowl [55], the mallard duck [20], in parrots [24], and in the anteriormost region of the principal piece of ratite spermatozoa [1, 3, 51, 52] but are described as "tiny" for the rhea, are absent from the tinamou [1], and are greatly reduced in columbiforms (Fig. 1S). They are present in suboscine and the more apomorphic oscine passerines, being larger in the latter. They are large and diverse in shape in marsupials above the didelphids, and in eutherian mammals (Fig. 1W). There thus appear to be trends to enlargement of the peripheral fibres in passerines and non-monotreme mammals, with diversification in the latter, and to reduction in ratites, and doves. In Chelonia, *Sphenodon*, *Caiman crocodylus* (but not apparently *Crocodylus johnstoni*) and in squamates, the fibres at doublets 3 and 8 are enlarged [16, 22] and are possibly homologous with the axial fibre, at 3, and juxta-axonemal fibre at 8, in lissamphibian sperm.

Significant synapomorphies are here suggested for amniote groups on the basis of the configuration of the peripheral axonemal fibres. In reptiles the peripheral fibres at 3 and 8 are detached from their corresponding doublets while the other seven fibres are attached to their doublets. In birds and monotremes all of the peripheral fibres are attached to the corresponding doublets [12]. In contrast to both of these assemblages, in therian mammal sperm the peripheral

Source : MNHN, Paris

fibres are detached from their doublets with the exception that fibres 3 and 8 may be close to or attached to their doublets [12, pers. obs.].

The peripheral fibres are usually situated in the midpiece with some extension into the principal piece as in turtles [22], the caiman [45], non-passerines [2], tuatara [22], and monotremes [7]. In the rhea and tinamou dense fibres are present only in the proximal principal piece. Very small dense fibres are present only in the distal region of the midpiece in the rooster and mallard; dense fibres in turtle dove sperm disappear before maturation is complete (see review by ASA & PHILLIPS [2]), though they persist through a short region of the midpiece in *Geopelia striata*. In eutherians and marsupials they extend far into the principal piece. However, in squamates, the only well developed, though small, peripheral fibres at the level of the annulus are the double fibres at doublets 3 and 8 and by the beginning of the principal piece all nine dense fibres are already vestigial or absent [27]. The fibres extend through most of the length of the sperm cell in oscine passerines [38].

In turtles, *Sphenodon* [16], *Crocodylus johnstoni*, and in skinks [13, 27, 28], the nine peripheral dense fibres are partly displaced from the radii of the triplets of the distal centriole into the gaps between adjacent triplets; the fibres are coradial with the doublets in the axoneme. These locations have been regarded as plesiomorphic for amniotes in Fig. 1. Dense material surrounds the central singlets in Chelonia (Fig. 1C), *Sphenodon* (Fig. 1G), and in crocodiles (Fig. 1K). In crocodiles the compact dense sheath appears to be a distinctive synapomorphy.

Snake sperm are characterized, apomorphically, by multilaminar membranes in place of the normal plasma membrane of the midpiece and axoneme [23, 35]; see also pygopodids [26].

The hypothetical plesiomorphic amniote spermatozoon (Fig. 2)

From the above comparative and cladistic considerations of the anatomy of amniote sperm, the following features may be attributed to the hypothetical plesiomorphic amniote spermatozoon. Relative to the tetrapod ground plan, deduced from common features of the amniote and lissamphibian sperm, amniotes are seen to have few basal synapomorphies. These are indicated, among plesiomorphic features of the amniote sperm, below.

The spermatozoon was elongate and filiform, with a hollow anterior conical acrosome vesicle overlying a simple subacrosomal cone. The base of the acrosome invested the tapered anterior tip (rostrum) of the nucleus and rested on pronounced nuclear 'shoulders'. The subacrosomal space within the acrosome contained two or three axial rods (putative perforatoria) or possibly only one rod. These penetrated the nucleus deeply, almost to its base, in endonuclear canals. At the base of the nucleus there was a compact fossa (implantation fossa) with which were associated two triplet centrioles of which the distal formed the basal body of the flagellar axoneme. The distal centriole was extremely elongate (amniote synapomorphy), traversing the entire length of the moderately elongate midpiece, the latter being defined by the presence of mitochondria. The mitochondria were subspheroidal, with concentric cristae and intramitochondrial dense body (amniote synapomorphies), and formed a circlet of several in cross sectional view around the distal centriole and several tiers in longitudinal section. The posterior end of the midpiece was defined by a subplasmalemmal dense annulus possibly homologous with the ring seen in urodele sperm [42]. The axoneme proper, consisting of nine peripheral doublet and two central singlet microtubles, posterior to the midpiece and constituting the principal piece, was surrounded by a fibrous sheath (amniote synapomorphy) which plesiomorphically was annulated. The terminal portion of the axoneme formed a short endpiece defined by the absence of the sheath. The elongate distal centriole and, internal to the fibrous sheath, a long anterior region of the axoneme, was surrounded by nine dense peripheral fibres, one to each triplet or doublet respectively (an amniote synapomorphy relative to fibres at 3 and 8, only, in lissamphibians). In the principal piece, two fibres, at doublets 3 and 8, were aligned with an inward projection

Source : MNHN, Paris

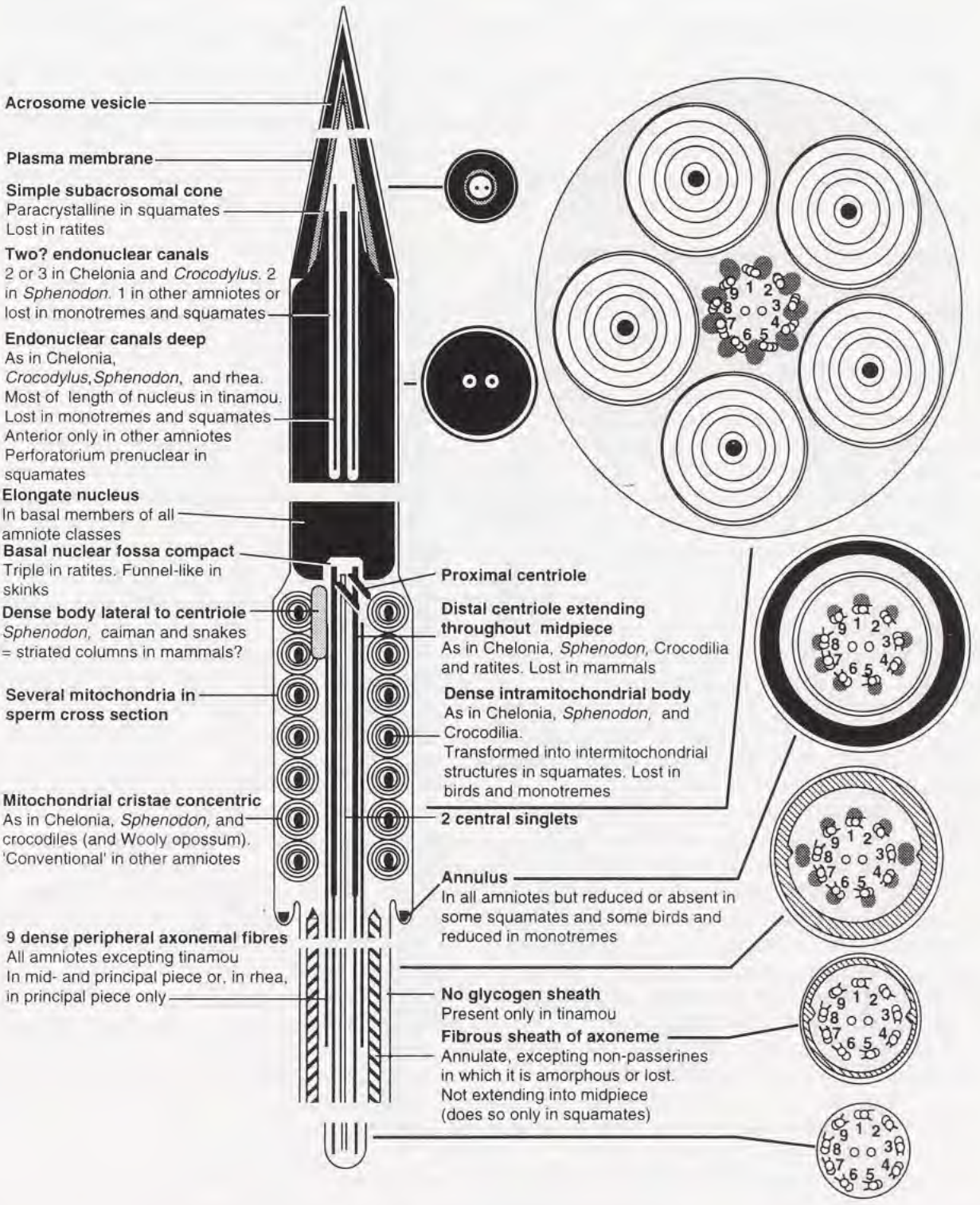


FIG. 2. — Diagrammatic representation of the hypothetical plesiomorphic amniote spermatozoon, the features of which are deduced in the text.

Source : MNHN, Paris

(longitudinal column) of the fibrous sheath. It is possible that the two fibres were enlarged and laterally displaced, and that all fibres in the centriolar region intruded into the inter-triplet radii, as in 'lower' amniotes (Chelonians, *Sphenodon* and crocodiles). As nine peripheral fibres are seen in lampreys and *Pantodon*, it might be considered that nine is the basic tetrapod, rather than merely amniote, number and that amphibians have lost all but those represented by the fibres at doublets 3 and 8 but there is no evidence in extant Lissamphibia for such a reduction and the presence of only two lateral elements in dipnoans and *Latimeria* suggests that nine fibres were an amniote synapomorphy, albeit homoplasic with some fish taxa. The retronuclear body seen in dipnoans and urodeles was lost or possibly transformed into the striated pericentriolar material.

The hypothetical plesiomorphic lissamphibian spermatozoon (Fig. 3)

A survey of the literature, of which only a few references can be given here, together with personal observations, on the sperm of urodeles [42], Gymnophiona [56], and Anura [25, 31, 32, 43] permits the following generalizations as to the ultrastructure of the sperm of ancestral lissamphibians (Fig. 3).

The hypothetical plesiomorphic lissamphibian spermatozoon may be attributed an anterior acrosomal vesicle forming a hollow, cone which overlay a cone of subacrosomal material. This embraced the tapered anterior end (rostrum) of the elongate nucleus. In Anura, basically, the acrosome vesicle and underlying cone embrace the nuclear rostrum. Urodeles (at least salamandrids and plethodontids, pers. obs.) are apomorphic in that the posterior limit of the acrosome vesicle is anterior to the nucleus, only the subacrosomal cone investing the nuclear rostrum. At the posterior end of the acrosome (vesicle and subacrosomal cone) the lissamphibian sperm nucleus plesiomorphically forms characteristic 'shoulders' posterior to which its form is cylindrical. Axially, within the acrosome, there is a rod, the putative perforatorium, which deeply penetrates the nucleus within an endonuclear canal. An axial rod (perforatorium), though lodged posteriorly in a much shorter endonucler canal, is also present in gymnophionids. As it is also present in the primitive frogs *Discoglossus* [43] and *Ascaphus* [25] and in the bombinids *Bombina* and *Alytes* [43], it is reasonable to deduce that a long acrosome rod and endonuclear canal is plesiomorphic for the Lissamphibia. This is endorsed by persistence of this condition in lower amniotes, the Chelonia, Sphenodontida and at least *Crocodylus johnstoni* in the Crocodilia. The base of the nucleus was indented as a basal nuclear fossa which contained the proximal centriole behind which lay the distal centriole which formed the basal body of the axoneme. Behind this, retronuclear material may have persisted to form the neckpiece but not as well developed as that seen in urodeles [42] above the cryptobranchs.

The structure of the sperm tail or flagellum is highly distinctive of the Lissamphibia. The axoneme has the structure usual for eukaryotes of nine peripheral doublet and two central singlet microtubules but it has distinctive appurtenances. To the generalized lissamphibian sperm can be attributed two longitudinal fibres, one on each side of the axoneme, adjacent to doublets 3 and 8. The fibre at doublet 8 is closely adjacent to the ring of doublets and may be termed the juxta-axonemal fibre at 8. This fibre is usually, but secondarily, absent in Anura. The fibre associated with doublet 3 is separated from the axoneme by a thin sheet of cytoplasm, the undulating membrane (with or without intervention of a juxta-axonemal fibre), and is termed the axial (major) fibre. The epithet axial refers to reported undulation of the axoneme around this stiff fibre in the Urodela and some primitive frogs (see KWON & LEE, this volume).

In urodele sperm, the axial fibre, at doublet 3, is connected to the axoneme by the undulating membrane but, typically, as in *Taricha granulosa* (Fig. 4C), there is no intervening juxta-axonemal fibre [4, 42]. *Plethodon albagula* [25, 32] is exceptional, of urodeles studied, in having a density on the adaxonemal end of the undulating membrane but it is questionable, because of its connection to dense bodies near the major fibre rather than to the fibre, that this is

Source : MNHN, Paris

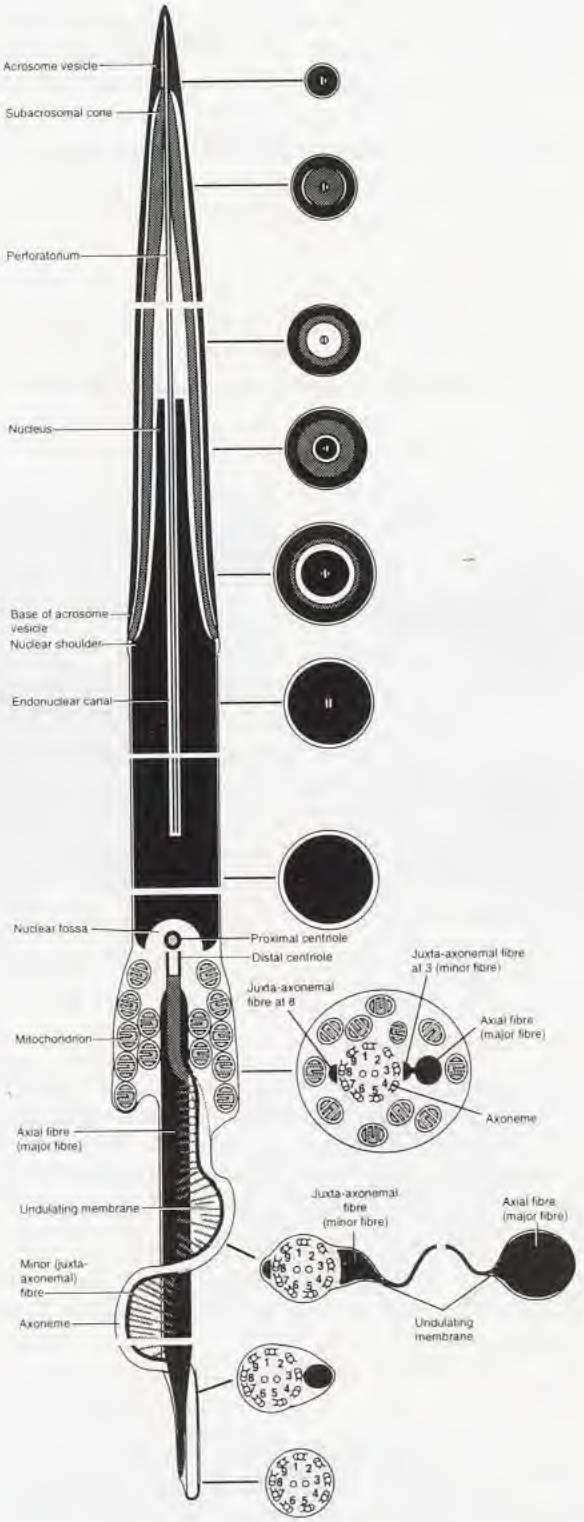


FIG. 3. — Diagrammatic representation of the hypothetical plesiomorphic lissamphibian spermatozoon, the features of which are deduced in the text.

Source : MNHN, Paris

homologous with the anuran juxta-axonemal fibre at 3. An axial fibre with undulating membrane is present in caecilians, in which juxta-axonemal fibres are absent [56]. Presence of a longitudinal fibre, near doublet 3, connecting to the axoneme via an undulating membrane appears, at least on first analysis, to be a synapomorphy of the Lissamphibia irrespective of whether it is subdivided into an axial (major) and juxta-axonemal (minor) fibre or is accompanied by a fibre at doublet 8. The possibility has been mooted [21, 25] (see also next section) that it is the *unilateral* location of the undulating membrane and its axial fibre, rather than presence of undulating membranes *per se*, which constitutes the synapmorphic condition for the Lissamphibia.

Pre-tetrapod presence of undulating membranes

Two longitudinal elements occur, one at each of doublets 3 and 8, in the dipnoan *Neoceratodus forsteri* (Fig. 4A) and the actinistian *Latimeria*. Sarcopterygian fish, and particularly dipnoans, appear to be the nearest extant non-tetrapod relatives of amphibians. Two, bilateral, elements which also occur at doublets 3 and 8 in Chondrichthyes are deduced to have been convergently acquired [21].

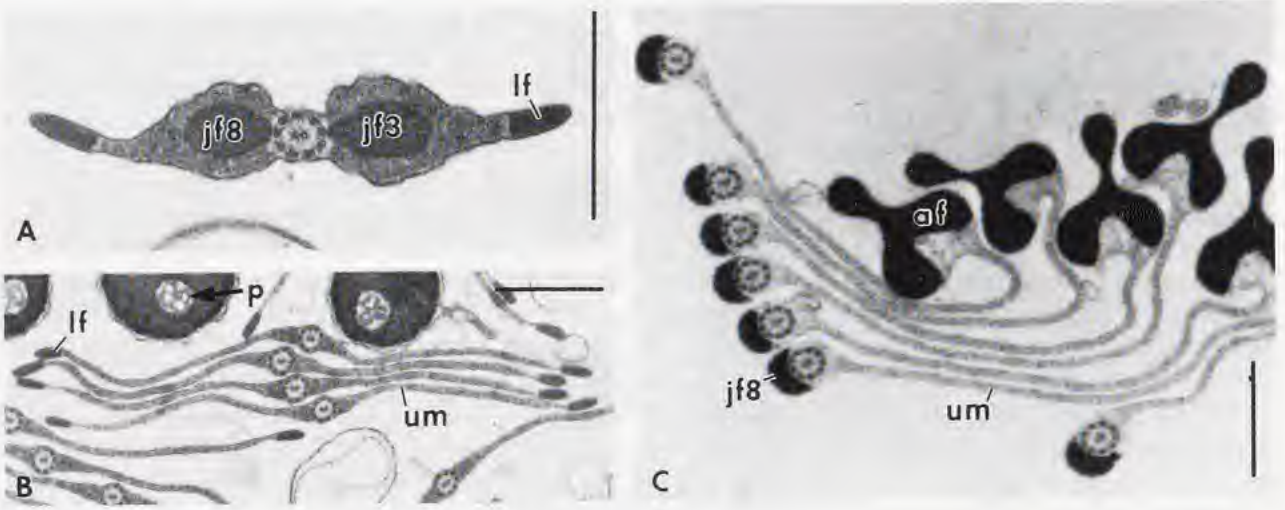


FIG. 4. — **A:** *Neoceratodus forsteri*. Transverse section through the anterior region of the tail of the spermatozoon. There is a large dense rod or fibre on each side of the axoneme, at doublets 3 and 8, and each rod is continuous with a 'fin' which terminates with a smaller, lateral density or fibre. **B:** Transverse sections of the axonemes more posteriorly where the fin is sufficiently extensive to be termed an undulating membrane. Three transverse sections of nucleus and endonuclear canals with contains perforatoria are also seen in the top of the figure. **C:** *Taricha granulosa*. Transverse sections through sperm tails showing urodele features of a longitudinal juxta-axonemal fibre at doublet 8, a long undulating membrane, and, in salamandroids, as in plethodontids, the Y-shaped transverse section of the axial (major) fibre. Abbreviations: af, axial (major) fibre; jf3 and jf8, juxta-axonemal fibres at doublets 3 and 8, respectively; lf, lateral fibre; p, perforatoria in the endonuclear canal of the nucleus; um, undulating membrane. Scale bar = 1 μm.

In *Neoceratodus* the anterior region of the sperm axoneme (not merely within the cytoplasmic canal as previously reported [29]) has a large dense rod on each side, at doublets 3 and 8, and each rod is continuous with a 'fin' which terminates with a further, smaller density (Fig. 4A, B). It is tempting to recognize homology between each fin and an amphibian sperm undulating membrane, between the large dense rods at doublets 3 and 8 and the amphibian juxta-

Source : MNHN, Paris

axonemal fibres and between the terminal densities (lateral fibres in Fig. 4A, B) and the axial fibre. This, if valid, would suggest that lissamphibians have retained, from an ancestor shared with dipnoans (and with other sarcopterygian fish?), only one of two former, bilateral, undulating membranes, and only one of a former pair of axial rods, but that the two juxta-axonemal fibres of urodeles are a persistence of the paired ancestral condition, the fibre at doublet 8 normally being lost in the Anura.

The condition in ray-finned fishes (Actinopterygii) of two lateral fins (also at doublets 3 and 8) [21] could conceivably have been precursory to lissamphibian undulating membrane and to dipnoan fins but homoplasy cannot be ruled out as lateral axonemal fins occur also in some echinoderms and protostomes.

Ground plan of the ancestral tetrapod spermatozoon

To the ground plan sperm of the basal tetrapod can be attributed all of the features which have been ascribed above to the ground plans of the lissamphibian and amniote spermatozoa with the exclusion of the synapomorphies identified for each of these groups. Synapomorphies of the tetrapod sperm relative to a presumed common ancestor shared with dipnoans, as exemplified by *Neoceratodus*, are deduced to have been few: nuclear 'shoulders' were developed as in basal amniotes and lissamphibians; an annulus (persisting in amniotes and possibly as the 'ring' in urodeles in the lissamphibians) may have developed or, alternatively, may have been retained from a pre-dipnoan ancestry. There were probably two longitudinal elements (dense fibres) peripheral to the axoneme adjacent to doublets 3 and 8, as in dipnoans, but these were apomorphically more extensive or, at least, well developed over a greater length, traversing much of the length of the axoneme. As noted above, a synapomorphy relative to *Neoceratodus* may have been loss of an undulating membrane between the fibre at 8 and the axoneme, leaving an albeit initially short undulating membrane between the fibre at 3 and the axoneme. The fibre at 8 would have persisted close to the axoneme. Plesiomorphic features deduced for the tetrapod sperm ground plan are the short distal centriole, and mitochondria, probably few immediately behind the nucleus, which had linear cristae as in lissamphibians. It appears unlikely that there were nine dense fibres as in amniotes.

Synapomorphies of higher amniote taxa

Chelonia and Sphenodontida. Turtles and *Sphenodon* conform to the hypothetical plesiomorphic amniote sperm and no convincing apomorphy seems demonstrable for the sperm of *Sphenodon* relative to the Chelonia.

Crocodilia. The ground plan for the Crocodilia, as exemplified by *Crocodylus johnstoni*, is very similar to that of the Chelonia and *Sphenodon*. All three have two or more endonuclear canals and concentric cristae with intramitochondrial bodies. Reduction to one perforatorial rod in *Caiman crocodylus* and *Crocodylus johnstoni* and restriction of the endonuclear canal to the anterior region of the nucleus in *Caiman* (though requiring confirmation) are apomorphies of the Crocodilia relative to Chelonia and *Sphenodon*. However, a possible synapomorphy of crocodiles, seen in *Caiman crocodylus* [45] and *Crocodylus johnstoni* (Fig. 1K) is investment of the two central singlets of the axoneme or of the distal centriole in a thick dense sheath which differs from the density, resembling a fibre, associated with the singlets in Chelonia, *Sphenodon* [16] and (homoplasically?) snakes [23, 35].

Aves. Loss of the subacrosomal cone may be a synapomorphy of birds as a whole. If they are the sister-group of crocodiles, they have apomorphically lost the concentric mitochondrial cristae. Ratites, though forming a monophyletic clade, have appeared paraphyletic [22] relative to non-ratite birds+monotremes but the three synapomorphies unifying the latter clade were insubstantial and probably artefactual. Monophyly of ratites cannot be considered proven,

Source : MNHN, Paris

however, as features considered to unify them (conical acrosome, fibrous sheath, and elongate centriole) [3] are all symplesiomorphies. Those in the earlier, cladistic analysis (supposed reversion to a long endonuclear canal, loss of the subacrosomal cone) [22] were also unconvincing and were associated with a computed paraphyletic origin of birds, in which ratites appeared as the sister group of galliforms+monotremes. If, however, the long endonuclear canal in more primitive ratites is a plesiomorphy carried over from basal amniotes, restriction of the canal to the anterior region of the nucleus in other non-passerines and passerines may be a synapomorphy of these, albeit homoplasic with crocodiles and derived ratites (emu). The avian feature of adhesion of all nine dense fibres to their axonemal doublets is also seen in monotremes; that it is apomorphic is debatable.

Squamata. Squamates are unified and distinguished by striking spermatozoal synapomorphies: loss of endonuclear canals and restriction of the perforatorial rod to a prenuclear location; development of intermitochondrial bodies (apparently from intramitochondrial bodies of lower amniotes); forward extension of the fibrous sheath into the midpiece; probably the paracrystalline structure of the subacrosomal cone; and, homplasically with other groups, shortening of the centriole from the elongate basal amniote condition.

Mammalia. Loss of the perforatorium (and endonuclear canal), homoplasic with some non-ratite birds, and great reduction of the distal centriole are synapomorphies jointly diagnosing mammal sperm. Separation, in marsupials and eutherians, of the peripheral dense fibres from their doublets with the exception that those at 3 and 8 may be attached to their doublets (the reverse of the situation in 'reptiles'), may be a therian synapomorphy.

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Source : MNHN, Paris

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Source : MNHN, Paris

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Source : MNHN, Paris

The Ultrastructure of Spermatozoa of the Squamata (Reptilia) with Phylogenetic Considerations

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ABSTRACT

Comparative ultrastructure of squamate families is reviewed, with new data for the South African chamaeleon, *Bradypodion karroicum*. Parsimony analysis is conducted, using Chelonia as the outgroup and branch and bound searching. Two major spermatozoal autapomorphies for the Squamata are extension of the fibrous sheath into the midpiece and (not computed) the paracrystalline subacrosomal cone. Further synapomorphies defining the Squamata *sensu strictu* are a single perforatorium in place of the two or three of Sphenodontida and Chelonia; loss of the endonuclear canal; presence of sinuous mitochondria (possibly an artefactual parsimony resolution as a columnar form is intuitively preferred); intermitochondrial location of dense bodies (mitochondrial transformations); presence of a well developed epinuclear electron lucent region and, equivocally, arrangement of the dense bodies as periodic rings. A major inference is polyphyly of the 'Sauria', the Scincomorpha and the Scincidae. Sphenomorphus group and egernid skinks show no close relationship to Eugongylus-group skinks which form the sister-group of the pygopodid *Lialis*. Snakes are the sister-group of the Eugongylus+pygopod clade. Gekkonidae appear to be a relatively plesiomorphic group, separated by several families from the Pygopodidae. The Iguania is not a monophyletic assemblage, iguanids and *Pogona* occur in the same clade but *Pogona* appears to be the sister-taxon of *Varanus*. Another iguanian, the chamaeleon *Bradypodion*, has an unresolved relationship with the gekkonid+snake+pygopodid+Eugongylus-clade. Sphenomorph and egernid skinks form an unresolved clade with *Chalcides* and lacertids but linkage of lacertids with the Teiidae in the Lacertoidea is not upheld. Pending further investigations of a larger number of taxa, these results can only be considered heuristic.

RÉSUMÉ

L'ultrastructure des spermatozoïdes des Squamata (Reptilia) et considérations phylogéniques

Ce chapitre comprend une synthèse de l'ultrastructure comparée des familles de Squamata, avec des observations nouvelles sur le Caméléon d'Afrique du Sud *Bradypodion karroicum*, et une analyse de parcimonie utilisant les Chelonia comme outgroup et une recherche par branch and bound. Les deux autapomorphies majeures des spermatozoïdes pour les Squamata sont l'extension de la gaine fibreuse dans la pièce intermédiaire et (n'intervenant pas dans le calcul) le cône subacrosomien paracristallin. D'autres synapomorphies définissant les Squamata *sensu strictu* sont un perforatorium unique à la place de deux ou trois chez les Sphenodontida et les Chelonia; la perte du canal endonucléaire; la présence de mitochondries sinueuses (ce qui pourrait donner lieu à un artefact de résolution car la forme en colonne est préférée de manière intuitive); la position intermitochondriale des corps denses (transformations mitochondriales); la présence d'une région épinucléaire claire aux électrons bien développée, et, de manière équivoque, la disposition des corps denses en anneaux périodiques. Une conséquence majeure est la polyphylie des "Sauria", des Scincomorpha et des Scincidae. Le groupe de Sphenomorphus et les Scincidae ergenides ne montrent pas de relations proches avec les Scincidae du groupe de Eugongylus, qui forment le groupe frère du pygopodide Lialis. Les Serpents sont le groupe-frère du clade

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Source : MNHN, Paris

Eugongylus+pygopodes. Les Gekkonidae semblent être un groupe relativement plésiomorphe, séparé par plusieurs familles des Pygopodidae. Les Iguania ne sont pas un assemblage monophylétique, les iguanides et *Pogona* se trouvent dans le même clade mais *Pogona* semble être le groupe-frère de Varanus. Les relations d'un autre iguanien, le caméléon *Bradypodion*, ne sont pas résolues avec le clade gekkonides+serpents+pygopodides+Eugongylus. Les Sphénomorphes et les Scincidae egernides forment un clade non résolu avec Chalcides et les lacertides mais la liaison des lacertides avec les Teiidae dans les Lacertoidea n'est pas soutenue. En attendant d'autres études sur un plus grand nombre de taxons, ces résultats peuvent seulement être considérés comme heuristiques.

There is no comprehensive well-corroborated phylogeny available for the Squamata [28]. As the utility of spermatozoal ultrastructure as a source of characters for phylogenetic analysis is well established [26, 27, 31, 32], JAMIESON *et al.* [37] and OLIVER *et al.* [45] attempted to shed light on squamate classification and phylogeny by a comparative study of spermatozoal ultrastructure which is reviewed and extended here.

The ultrastructure of spermatozoa or spermiogenesis has been studied, though often cursorily, in the major groups of squamate reptiles. Families studied are: Scincidae [8, 14, 21, 29, 34, 35, 37, 44]; Lacertidae [7, 11, 21]; Teiidae [19, 41, 42]; Iguanidae [22, 48]; Anolidae [10]; Tropiduridae [12, 13]; Agamidae [2, 9, 15, 16, 17, 18, 45]; Chamaeleonidae [52 (spermiogenesis only), and this account]; Varanidae [45]; Gekkonidae [21, 37, 46]; and Pygopodidae [25, 37]. A brief account of the kinetic apparatus of the sperm of *Amphisbaena darwinii* (Amphisbaenidae) and *Liolaemus weigmanii* (Iguanidae) by SOTELO & CENÓZ [50] is chiefly of historic interest and will not be reviewed here. The spermatozoa of Serpentes have been more thoroughly investigated, in terms of taxa examined, than those of other squamates [1, 3, 5, 6, 20, 21, 24, 33, 45, 46, 49].

The present account reviews squamate sperm, provides the first description of mature chamaeleonid sperm, for the rare South African chamaeleon *Bradypodion karroicum*, and gives a preliminary parsimony analysis.

MATERIALS AND METHODS

Testes and ducts were dissected from a euthanased specimen of *Bradypodion karroicum* (Chamaeleonidae). Processing of the tissues was as in [34]. A cladistic analysis was performed using the PAUP program of SWOFFORD [51] (for details see parsimony analysis).

RESULTS AND DISCUSSION

Comparative sperm ultrastructure

Spermatozoal ultrastructure in the Squamata is summarized in Tables 1 and 2.

Scincidae. Descriptions of the male gametes of the Scincidae include a description of the mature spermatozoon of *Chalcides ocellatus tiligugu* [21]; an account of spermiogenesis, with some description of mature, epididymal sperm, in the same subspecies [8]; brief descriptions of spermiogenesis in *C. ocellatus* [14, 29]; an account of development of the midpiece in *Eumeces laticeps* [44]; and descriptions, with phylogenetic considerations, by JAMIESON & SCHELTINGA, of the sperm of the sphenomorph skink *Nangura spinosa* [34] and of *Tiliqua scincoides*, and *Ctenotus taeniolatus*, with brief reference to *Anomalopus verreauxii* [35]. JAMIESON *et al.* [37]

FIG. 1. — A generalized spermatozoon (diagrammatic), in longitudinal and corresponding transverse sections, of the Sphenomorphus group of the Scincidae (*Nangura spinosa*, *Ctenotus taeniolatus*, *C. robustus*, *Anomalopus verreauxii*) and Egernia-group (*Tiliqua scincoides scincoides*). Scales of various components are only approximate. Regional zonation of the acrosome vesicle is shown only for the transverse sections. After [34, 37].

Source : MNHN, Paris

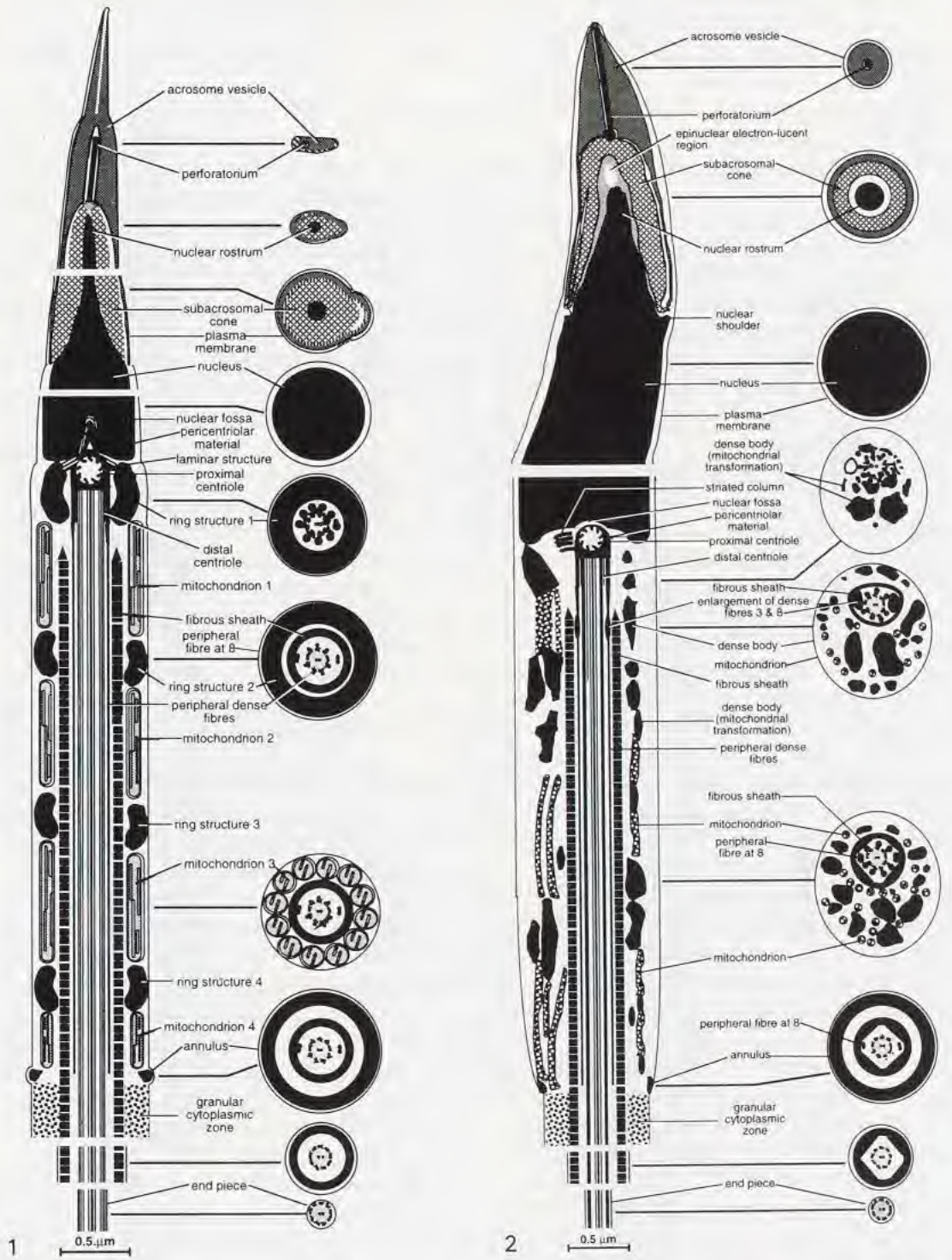

FIG. 2. — A generalized spermatozoon (diagrammatic) of the Eugongylus-group species of the Scincidae, in longitudinal and corresponding transverse sections. It is drawn from the sperm of *Carlia pectoralis* but is applicable also to *Cryptoblepharus virgatus* (Eugongylus-subgroup), and *Lampropholis delicata* (with *Carlia*, in the Lampropholis-subgroup). Scales of various components are only approximate. Regional zonation of the acrosome vesicle, though present, is not indicated. After [37].

Source : MNHN, Paris

have compared the sperm of *Ctenotus robustus,* a scincid of the Sphenomorphus group and other sphenomorphs (*Ctenotus taeniolatus*, *Nangura spinosa*), with those of *Cryptoblepharus virgatus*, *Lampropholis delicata*, and *Carlia pectoralis*, in the Eugongylus-group, in the classification of GREER [23].

Spermatozoa of skinks (e.g. *Ctenotus robustus*, *Carlia pectoralis*, *Cryptoblepharus virgatus*, and *Lampropholis delicata*) conform with other squamate sperm in the following respects: the sperm are filiform; the acrosome vesicle is in the form of a hollow, concentrically zoned cone which basally overlies a paracrystalline subacrosomal cone which invests the tapered anterior end of the nucleus; the perforatorium is a slender rod extending anteriorly from the subacrosomal material; the midpiece terminates with an annulus; peripheral dense fibres are associated with the 9 triplets of the distal centriole and the doublets of the axoneme within the midpiece; the peripheral fibres adjacent to doublets 3 and 8 are enlarged and each forms a double structure associated with the annulated fibrous sheath; usually all nine peripheral fibres are absent from the principal piece though in *Lampropholis delicata* they remain well developed in its anterior region and fibres 3 and 8 sometimes extend into the endpiece; the fibrous sheath extends anteriorly into the midpiece (squamate autapomorphy).

The sperm of species of the Sphenomorphus group (e.g. *Ctenotus*) and the Egernia-group (*Tiliqua*) (Fig. 1) differ from Eugongylus-group species (*Cryptoblepharus virgatus*, *Lampropholis delicata* and *Carlia pectoralis*) (Fig. 2), in the classification of GREER [23], in the following features: (1) the acrosome is elongate (it is relatively short in Eugongylus-group species); (2) the acrosome is depressed near its tip; (3) the perforatorium is strongly oblique (it is very slightly oblique in Eugongylus-group species); (4) a conspicuous laminated structure is present on each side of the proximal centriole (it is absent, though possibly represented by striated column(s) in Eugongylus-group species); (5) the midpiece is shorter absolutely and relative to the nucleus; (6) the midpiece has four dense ring structures in longitudinal succession (in Eugongylus-group species mitochondrial transformations are scattered irregular dense bodies of varying sizes); (7) mitochondria between the mitochondrial transformations form columnar structures in a circle around the fibrous sheath with numerous predominantly longitudinal cristae (in Eugongylus-group species mitochondria are elongate, tubular structures, with indistinct cristae, and weave between the intermitochondrial bodies); (8) enlargement of the peripheral fibres adjacent to doublets 3 and 8 occurs, as in all squamates, but not the gross enlargement which occurs in the anterior region of the axoneme in *Carlia* and *Lampropholis*.

From microcomplement fixation of albumin, BAVERSTOCK & DONNELLAN [4] showed the Eugongylus-group to be monophyletic as suggested by sperm ultrastructure [35].

The sperm of the European scincid species *Chalcides ocellatus tiligugu* [21] conforms closely to the description given above for the Sphenomorphus group, particularly in having a longitudinal series of four dense rings alternating with columnar mitochondria. An annulus was not described but absence is doubtful in view of its presence in all squamates which have been examined by the author. *Ch. ocellatus tiligugu* differs from the Sphenomorphus group in the composition of the dense rings which are shown diagrammatically as each being composed of large juxtaposed granules in single file. CARCUPINO *et al.* [8] describe the ring structures as "four rings of electron-dense material" and do not mention a granular composition.

For the scincid *Eumeces laticeps*, OKIA [44] described a midpiece with nine mitochondrial columns around the axoneme, a condition reminiscent of that in sphenomorphs. These "columns lie segmented by partial or complete rings of dense material" but it is not clear whether their arrangement conforms to the sphenomorph pattern.

A suite of character states which JAMIESON & SCHELTINGA [35] noted for the Sphenomorphus and Egernia-groups of the Scincidae is also seen in the teiid lizard, *Cnemidophorus sexlineatus* [41, 42] (see below): 1. Anterior depression of the acrosome. 2. The conical nuclear fossa containing dense material projecting from the proximal centriole. 3. The

Source : MNHN, Paris

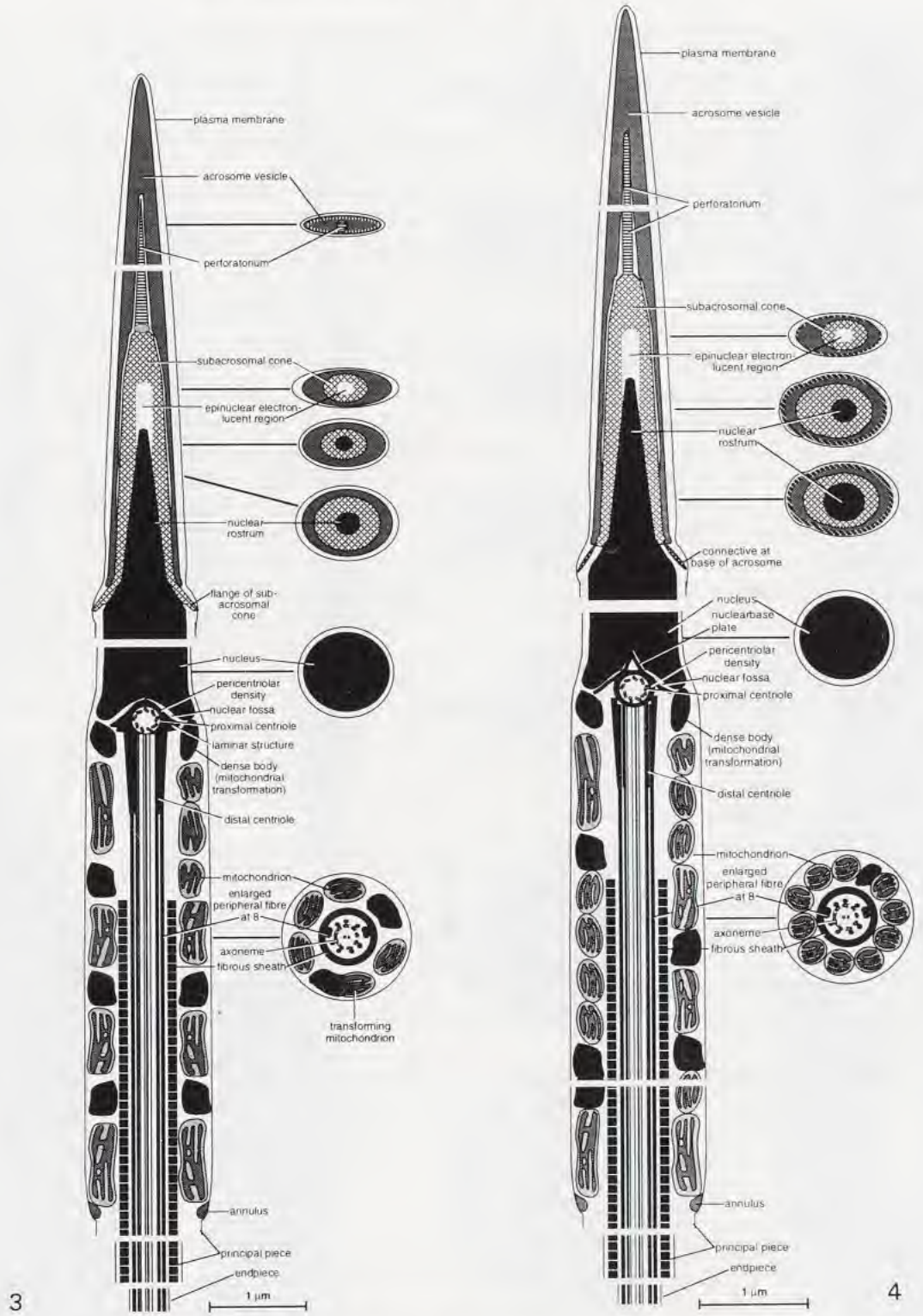

FIG. 3. — *Pogona barbata* (Agamidae). A diagrammatic representation of the spermatozoon, in longitudinal and corresponding transverse sections. After [37].

FIG. 4. — *Bradypodion karroicum* (Chamaeleonidae). A diagrammatic representation of the spermatozoon, in longitudinal and corresponding transverse sections. Original.

Source : MNHN, Paris

presence of a laminar structure extending from the pericentriolar apparatus (possibly unilateral in the teiid, and possibly homologous with the striated column(s) in Eugongylus-group sperm). 4. Presence of four, or in *Cnemidophorus* five, intermitochondrial rings alternating with columnar mitochondria. 5. The absence of sharply defined nuclear shoulders. 6. The apparent wide separation of the plasma membrane from the fibrous sheath in the anterior region of the principal piece. However, features 3 and 6 require confirmation for teiids.

The parsimony analyses (below) which are necessarily preliminary in view of our imperfect knowledge of these and other characters across the spectrum of squamate taxa, strongly suggest that skinks are not monophyletic and that sphenomorph-egernid and Eugongylus-group skinks are two distinct groups of squamates, the Eugongylus-group appearing to be the sister-group of pygopods (Fig. 10). As snakes form the sister-taxon of these two groups, it would appear that the legless condition has been independently derived in snakes as compared with pygopods, though as a parallelism from an ancestor shared also with Eugongylus-group skinks.

Lacertidae. Spermatozoa of the Lacertidae have been examined ultrastructurally in *Lacerta sicula campestris*, *L. lepida lepida*, *L. laevis*, *L. viridis* and *Algyroides alleni* [21], *L. vivipara* [11] and, with reference to development of the sperm head, *Podarcis* (=*Lacerta*) *taurica* [7]. They are morphologically very similar and resemble those of the sphenomorph-egernid Scincidae in many respects [21]. The head is curved and depressed [7, 11, 21]. The perforatorium ("apical groove") is oblique and the subacrosomal cone is paracrystalline [7, 21] as is typical of squamates. The midpiece is distinctive in having only two sets of dense bodies, the first has the appearance of two opaque masses on each side immediately posterior to the nucleus [21] and termed the "nuclear plate" [11]; this is probably correctly regarded by NEWTON and TRAUTH [42] as the equivalent of the first ring structure in teiid sperm. The second is a large ring (more certainly equivalent to ring structures of sphenomorph skinks) and questionably regarded as a "chromatoid body" [11], between the mitochondria in the distal third of the midpiece. In *L. vivipara*, there are only three tiers of mitochondria in longitudinal section and the intermitochondrial ring lies between mitochondrial tiers 2 and 3 [11]. The appearance and size of the dense bodies varies from species to species but not clearly enough to be used as a defining characteristic. The internal surface of the posterior ring is indented by numerous niches containing small masses of moderately opaque material [21]. In the order of 10 mitochondria are illustrated in transverse section for *L. lepida* [21] but only five in *L. vivipara* [11]. Though said to be filamentous [21], the mitochondria appear compact and only a few times longer than wide, with linear cristae. Peripheral dense fibres at 3 and 8 are enlarged and fused with or adpressed to the fibrous sheath [11, 21]. In mature sperm no annulus is indicated diagrammatically but in a micrograph a very small, weakly developed annulus appears to be present [21], and it is present in the late spermatid [11]. There are two centrioles and the implantation fossa is compact and rounded [11, 21].

Teiidae. The subacrosomal rod (putative perforatorium) has been shown in *Cnemidophorus lemniscatus lemniscatus* to develop from a subacrosomal granule [19]. The spermatozoon of

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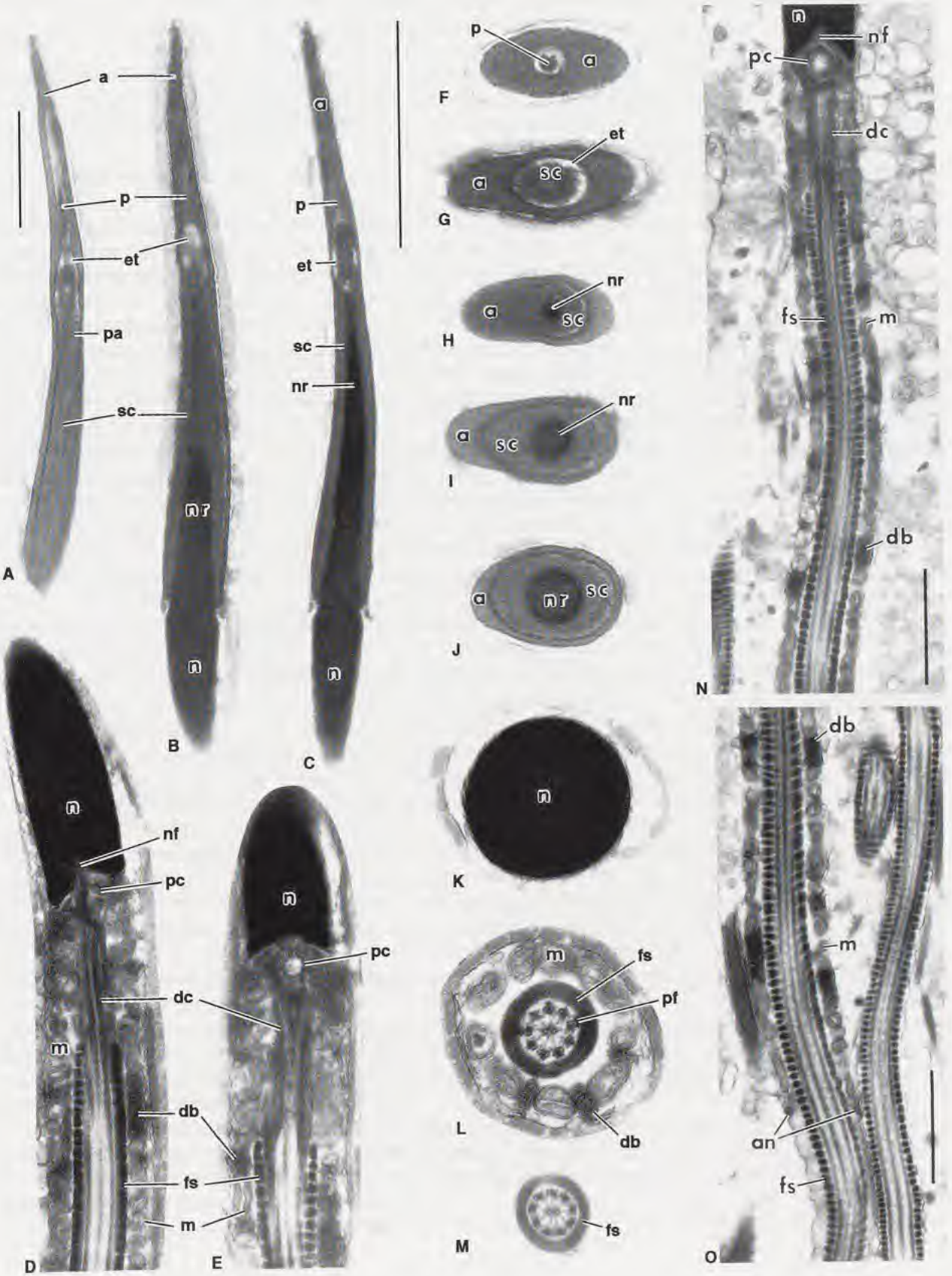
FIG. 5. — *Bradypodion karroicum* (Chamaeleonidae). **A, B, C:** Longitudinal sections (LS) of acrosome and anterior region of nucleus. **D, E:** LS of posterior region of nucleus and anterior region of midpiece. **F-M:** Successive transverse sections through F-J, the acrosome, K, the nucleus, L, the midpiece and M, the principal piece. **N:** LS centriolar region and midpiece. **O:** LS posterior region of midpiece, including annulus, and anterior region of principal piece. a = acrosome vesicle; an = annulus; db = dense body (mitochondrial transformation); dc = distal centriole; et = electron lucent space; fs = fibrous sheath; m = mitochondria; n = nucleus; nf = nuclear fossa; p = perforatorium; pc = proximal centriole; pf = peripheral dense fibre (coarse fibre); pm = plasma membrane; sc = subacrosomal cone. Original. Scale bar 1 µm.

Source : MNHN, Paris



Source : MNHN, Paris

C. sexlineatus has been the subject of detailed description [41, 42], augmented here by reference to published micrographs. A suite of characters shared with the Sphenomorphus and Egernia-group Scincidae is discussed under that family, above.

In *C. sexlineatus*, the acrosome caps and invests the anterior 1 μm of the nucleus which forms a pointed nuclear rostrum. The acrosome is depressed and spatulate, as in Sphenomorphus and Egernia-group scincids. Although an acrosome vesicle is recognized, no subacrosomal cone is described. Instead, a large vesicle and a more basal small vesicle are described posterior to the main part of the acrosome vesicle. From a micrograph there seems some possibility that the large and small vesicles represent subacrosomal material. It appears that a basal extension of the acrosome vesicle envelops this material, as in typical squamate and, indeed, tetrapod spermatozoa. This interpretation is supported by presence of an electron dense connective between the posterior acrosome and the cell membrane. A well developed rodlike perforatorium, composed of longitudinal fibres, possibly in helical array, is present. It does not appear to be oblique but is unusual in being strongly eccentric. A narrow epinuclear electron lucent zone is visible anterior to the rostrum. The nucleus is curved and strongly condensed and has a low, conical, basal implantation fossa. The absence of sharply defined nuclear shoulders at the base of the rostrum is a resemblance to the above-mentioned scincids. The midpiece consists of five tiers, usually, of shortly columnar mitochondria separated by intermitochondrial dense "ring structures" and terminating with an annulus, giving the formula rs1/mi1, rs2/mi2, rs3/mi3, rs4/mi4, rs5/mi5, an. Each mitochondrial set consists of 8 to 10 (usually 9) mitochondria arranged around the axoneme. The axoneme is surrounded by the fibrous sheath from rs3 to the posterior end of the principal piece, leaving a 0.7 μm endpiece. The A subtubules of the 9+2 axoneme are filled with dense material. A transverse section of the distal centriole (basal body) shows coarse fibres enveloping the triplets, extending both peripherally and internally to each triplet, and, as usual, displaced clockwise into the inter-triplet space. Dense material is associated with the two central singlets. Coarse fibres are absent from illustrated transverse sections of the principal piece. Longitudinal extracellular tubules surround the nucleus and flagellum in the ductus deferens. The suggested presence of a laminar structure extending from the pericentriolar apparatus, equivalent to the bilateral structure in scincids [35], requires confirmation.

Iguanidae. The account by FURIERI [22], based chiefly on three species of the Iguanidae, *Cupriguanus scapulatus*, *Phymaturus palluma* and *Liolaemus austromendocinus*, is summarized here, using the terminology employed in the present account. The general structure of the spermatozoon is much as described for skinks, with the following details. The acrosome is circular in cross section; the single, well developed perforatorium appears to have a pointed, not square, tip and to lack a basal plate but this requires confirmation. An endonuclear canal is absent. The subacrosomal cone ("inner cap") is paracrystalline and surrounds a pointed nuclear rostrum which is preceded by an epinuclear electron lucent region. The nucleus is elongate, with condensed chromatin, and has a shallow basal fossa which houses the anterior portion of the proximal centriole; this is at an angle to the distal centriole. There are distinct nuclear shoulders, apparently intermediate in shape between the sharp and rounded forms. The midpiece is relatively short, and, with a length of 7 μm, is much shorter than the head. Although the mitochondria, which are long, thin and numerous, are arranged in a regular circlet around the axoneme and fibrous sheath, they form helices, with a regular arrangement in *C. scapulatus* and especially *L. austromendocinus*. They have a very fine calibre in *L. austromendocinus*, thus being reminiscent of those of snake sperm. The quantity and arrangement of the dense intermitochondrial bodies are characteristic of each of the three species. In *Phymaturus*, the mitochondrial sleeve is subdivided into six long sections, separated by contiguous small masses of opaque material arranged in a ring which is sometimes not completely closed. A sixth dark ring is present at the beginning of the mitochondrial sleeve and a seventh, much smaller ring (here considered to be the annulus) closes the sleeve, giving the formula, in the system of NEWTON and

Source : MNHN, Paris

TRAUTH [42], of rs1/mi1, rs2/mi2, rs3/mi3, rs4/mi4, rs5/mi5, rs6/mi6, an. In *Cupriguanus*, the dense material is more scanty and does not seem to form closed rings. In *Liolaemus*, the dense bodies form small dark plates which are few in number and are distributed without order. Development of nine peripheral axonemal fibres is limited to the short region of the midpiece anterior to the fibrous sheath. Within the fibrous sheath there are only two, enlarged fibres and these are joined to the sheath. These are said to lie at doublets 3 and 7 but the micrographs and diagram indicate that they are in the usual position adjacent to doublets 3 and 8.

Anolidae. The account of spermiogenesis in the so-called American chamaeleon, *Anolis carolinensis* (Anolidae) [10] reveals no characters which can conclusively be ascribed to the mature spermatozoon. However, the spermatid has the usual acrosome vesicle and subacrosomal material investing the nuclear rostrum; a condensed, curved nucleus; a low, dome shaped but apically pointed implantation fossa; and a proximal centriole tilted relative to the distal centriole. The fibrous sheath is posterior to the annulus but whether it later extends into the midpiece is not known, though likely.

Tropiduridae. Accounts of spermiogenesis in *Tropidurus torquatus* [12, 13] yield little information with regard to the mature spermatozoon. The following account is derived from text and micrographs. As usual for squamates, the acrosome vesicle and underlying subacrosomal cone ensheath a tapered anterior extension of the nucleus (nuclear rostrum). Smooth nuclear shoulders support the posterior end of the acrosome. There is a large lacuna within the nuclear rostrum, near it tip. The sperm head does not appear to be depressed. The very short midpiece has only three gyres of mitochondria. The statement that the fibrous sheath is found only in the principal piece, except for a few dense spots around the annulus (unlike any other known squamate sperm) is to be doubted as the observation is not derived from a mature spermatozoon. Peripheral dense fibres are limited to a short segment near the short neck cylinder which lies between the proximal centriole and the base of the nucleus. Multiple cytoplasmic membranes around the acrosome tend to disappear by maturity whereas those in snakes and pygopods appear to persist. No evidence of dense bodies or ring structures is provided [13].

Agamidae. In the Agamidae, differentiation of the sperm head has been described for *Agama stellio* [2], *Uromastyx philbyi* [15], *Stenodactylus selvini* [16], for *A. adramitana* [18] and, for the nuclear manchette, *A. agama* [9]. Differentiation of the sperm tail has been described in *Uromastyx philbyi* [17]. The entire sperm has been described for *Pogona barbata* [45] (Fig. 3).

The mature spermatozoon of *Agama stellio* illustrated by AL-HAJJ *et al.* [2] conforms closely in ultrastructure to that of the cofamilial *Pogona barbata* [45]. Flattening of the acrosome is again seen [2], as in *A. adramitana* [18], but supposed flattening of the nucleus is not supported by micrographs. A well defined narrow acrosomal cortex is present (see also [8, 18]). From published accounts [37], division of the acrosome into cortex and medulla can be claimed not only for the Agamidae but also for Lacertidae, Teiidae, Chamaeleonidae, Varanidae and all families of the Serpentes. This subdivision is not apparent in Gekkonidae but it is possible that layering described for sphenomorph skinks [34] and the anterior saccular enclave seen in the Eugongylus-group skink *Lampropholis delicata* and in the pygopod *Lialis burtonis* [37] is equivalent. The wide zone between the acrosomal cortex and the perforatorium [2, 18] is clearly the subacrosomal cone. Late spermiogenic stages of *A. agama* [9] confirm the circular nuclear section but flattened acrosome and wide, distinct nuclear shoulders which, as in *Pogona barbata*, are intermediate between the concave, angular shoulders of, for instance, Eugongylus-group scincids, and the rounded shoulders of sphenomorphs.

With regard to affinities of the sperm of *Pogona barbata*, it shares a larger number of character states with the sperm of *Varanus gouldii* than with other non-agamid squamate taxa which have been studied. Those also common to the sphenomorph-teiid-varanid assemblage have been indicated above.

Source : MNHN, Paris

A varanid-agamid relationship does not appear to have been suggested on the basis of somatic morphology (see review by RIEPPEL [47]). Of the three noteworthy spermatozoal similarities of depressed acrosome, alternating, intermitochondrial rings and the not entirely similar basal plates, only the knob-like form of the basal plate appears to be an agamid-varanid synapomorphy and it is homoplasic with Eugongylus-groups skinks. Nevertheless, the validity of recognizing a varanid-agamid relationship deserves further consideration.

A number of features shared between *Pogona barbata* and sphenomorph skinks, *Tiliqua scincoides* (Egernia-group), *Cnemidophorus sexlineatus* (Teiidae) and *Varanus* appear, on intuitive consideration and in parsimony analysis, to be symplesiomorphic for these taxa: the single, pointed perforatorium and linear cristae (plesiomorphic for squamates); the epinuclear electron lucent zone, the elongate, cylindrical nucleus (plesiomorphic for tetrapods); absence of multilaminar membranes around the midpiece (presence has recently been recognized as a similarity and possible synapomorphy of snakes and pygopods [37, and this account]; and extension of the fibrous sheath into the midpiece (autapomorphy of squamates).

Chamaeleonidae. A preliminary study of the spermatozoon of the rare South African chamaeleon, *Bradypodion karroicum* (Figs 4, 5), reveals close similarity, on intuitive consideration, to the spermatozoa of the Agamidae (see above). The acrosome is sharply attenuated but is depressed in one plane, The acrosome vesicle is divisible into cortex and medulla. Within the acrosome medulla the subacrosomal space encloses a perforatorium in the form of a narrow cylinder which tapers to a blunt point anteriorly and is cross striated. A basal plate, questionably present in *Pogona barbata*, is absent. The subacrosomal cone, occupying the basal two thirds of the length of the acrosome, is conspicuously paracrystalline. The cylindrical, electron dense nucleus has slight, smoothly rounded shoulders and a long, slightly curved nuclear point (rostrum) which projects almost to the tip of the subacrosomal cone and is preceded by an electron lucent epinuclear region. There is a broad basal nuclear fossa which, in being pointed anteriorly, resembles that of sphenomorph skinks more closely than the shallow rounded fossa of *Pogona*. The proximal centriole, consisting of nine triplets, lies at right angles to the long axis of the sperm with its anterior region within the nuclear fossa (implantation fossa); a narrow density anterolateral to this centriole appears to continue to the summit of the fossa. As in agamids, a distinct lamellar structure is not discernible. As in *Pogona*, the distal centriole, forming the basal body of the axoneme, is elongate but similarly does not extend into the fibrous sheath. In transverse section of the midpiece through the fibrous sheath approximately 10 circular mitochondrial profiles form a single circlet around the sheath. In longitudinal sections, the profiles are again mostly circular but may show elongation consistent with a spiral arrangement and this is particularly true of the mitochondria in the short region of the midpiece anterior to the fibrous sheath. Small intermitochondrial dense bodies sporadically interrupt the mitochondria in transverse and longitudinal sections. Dense rings of the type seen in sphenomorph skinks are not present. A large dense granule is visible in some mitochondria and there is evidence of transformation of mitochondria into intermitochondrial dense bodies. The midpiece is moderately elongate and terminates at a distinct if small annulus. In the midpiece the axoneme has ninr coarse fibres; those at 3 and 8 are enlarged, appear double, and are adpressed to the fibrous sheath. No coarse fibres are visible in the principal piece, posterior to the mitochondrial midpiece. For a considerable distance posterior to the annulus a wide band of cytoplasm surrounds the fibrous sheath as in *Pogona barbata* and *Varanus gouldii* [45] but also in Eugongylus, sphenomorph, and egernid group skinks [35] and in the gekkonid *Heteronotia binoei* [37].

In the parsimony analysis (Fig. 10) chamaeleonids have an unresolved position, with gekkonids, at the base of the Eugongylus-group-pygopodid-snake clade. Their usual iguanian relationship or placement in the Agamidae is not upheld.

Varanidae. In view of the fact that only one varanid has been examined for spermatozoal

Source : MNHN, Paris

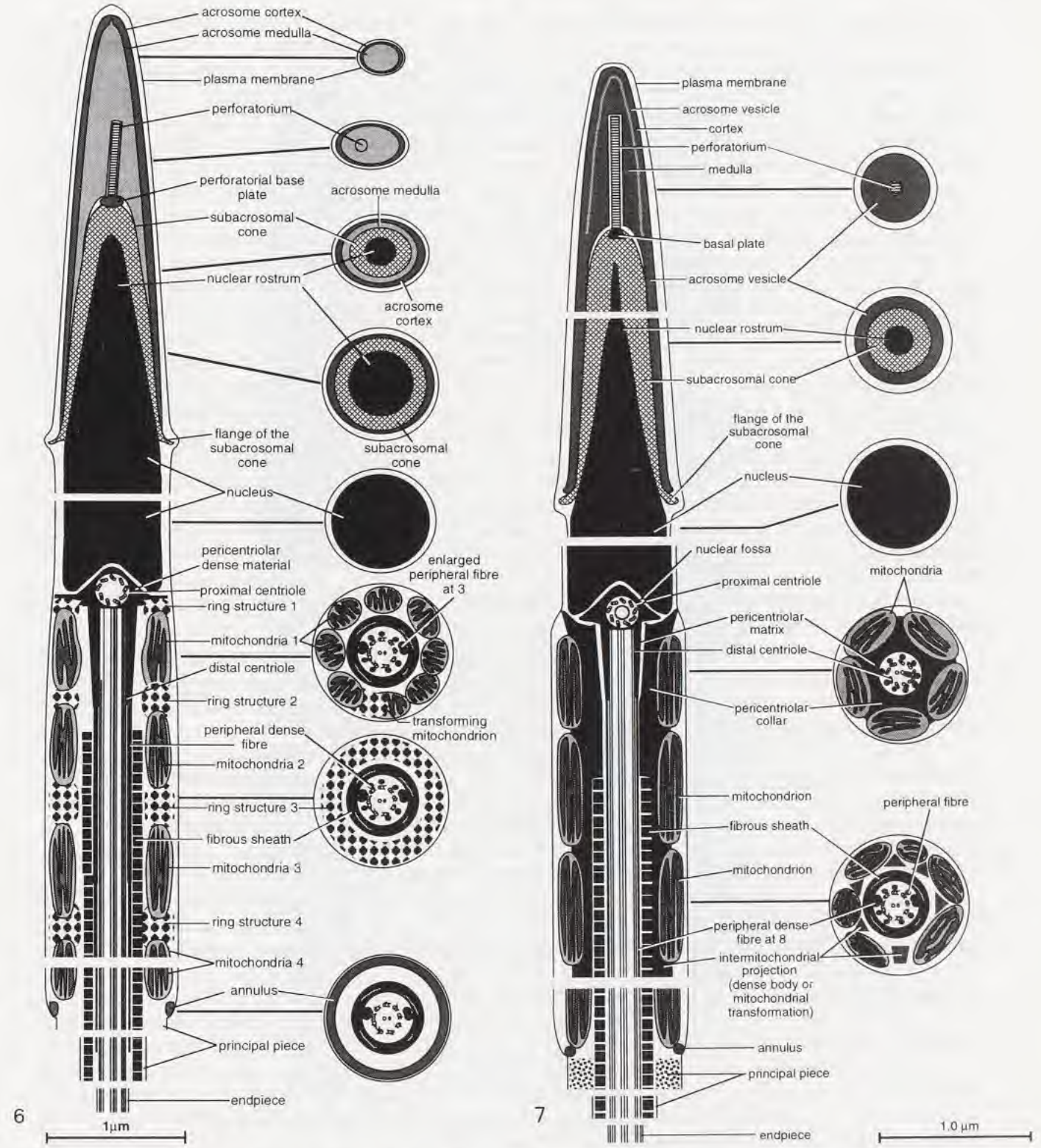


FIG. 6. — *Varanus gouldii flavirufus* (Varanidae). A diagrammatic representation of the spermatozoon, in longitudinal and corresponding transverse sections. After [45].

FIG. 7. — *Heteronotia binoei*. (Gekkonidae). A diagrammatic representation of the spermatozoon, in longitudinal and corresponding transverse sections. After [37].

Source : MNHN, Paris

ultrastructure (Fig. 6), the following comparison with the sperm of other squamates can be considered only a most preliminary indication of affinities of the family. A suite of character states has been described for the sperm of *Varanus gouldii flavirufus* [45] which is known elsewhere in squamates in the Sphenomorphus and Egernia (*Tiliqua*) groups of the Scincidae [37] and in the teiid lizard, *Cnemidophorus sexlineatus* [42] although some are also seen in agamids (e.g. *Pogona barbata* [45]). This suite includes the following states. 1. Anterior depression of the acrosome (also in agamids). 2. The conical nuclear fossa containing dense material projecting from the proximal centriole. 3. The presence of a laminar structure extending from the pericentriolar apparatus (possibly unilateral only in the teiid) although this is less definitely laminated in *V. gouldii*. 4. Presence of four intermitochondrial rings alternating with columnar mitochondria. The rings differ in *V. gouldii* in being composed of many large granules in contrast with the solid condensed structure in the sphenomorph-Egernia-group and in *Cnemidophorus*. The difference is lessened, however, by supposed constitution of the rings in the scincid *Chalcides ocellatus* from a single circlet of large granules [21]. (An alternation of mitochondria and albeit incomplete rings occurs in also in *P. barbata*). 5. The rounded rather than angular nuclear shoulders are apomorphic relative to the "sharp" or angular shoulders of *Sphenodon* [32] and primitive frogs [36]; a feature occurring only in the Eugongylus-group skinks [37]. (*P. barbata* shows an intermediate condition.) 6. The wide separation of the plasma membrane from the fibrous sheath in the anterior region of the principal piece (also in *P. barbata*), although this has not been demonstrated with certainty for *Cnemidophorus*.

In the parsimony analysis (Fig. 10) the alternation of ring structures with mitochondrial columns computes as an ambiguous basic synapomorphy of the squamates as a whole, rather than merely of the iguanid through sphenomorph assemblage. Flattening of the acrosome computes as the sole basal synapomorphy of this assemblage, independently developed in *Bradypodion*. Consideration may be given, however, to the possibility that flattening is basic to squamates and has been lost in the snake+pygopodid+Eugongylus-group clade.

Gekkonidae. In the Gekkonidae, FURIERI [21] briefly described the sperm of *Lygodactylus picturatus* and made reference to *Hemidactylus frenatus*, *H. mabouia*, and *Tarentola mauritanica mauritanica*; PHILLIPS & ASA [46] described formation of the midpiece in *Sphaerodactylus cinereus* and JAMIESON *et al.* [37] described the sperm of *Heteronotia binoei*.

In the spermatozoa of *H. binoei* (Fig. 7) no epinuclear electron-lucent region has been observed (computing as a loss); nuclear shoulders are smooth, as in sphenomorph skinks; mitochondria are large and discrete, arranged in a circle around the fibrous sheath, with intervening mitochondrial transformations, and extend longitudinally as slender columns. A feature not known in skinks is indentation of the median surfaces of the mitochondria at intervals by triangular dense bodies which are perhaps always longitudinally interconnected.

The sperm of *Lygodactylus picturatus*, *Tarentola mauritanica* and *Hemidactylus frenatus* [21] are generally similar to those of *H. binoei*, but the amount of intermitochondrial material (dense bodies or putative mitochondrial transformations of the present work) is greater in *Lygodactylus picturatus* and is present in decreasing amounts in *Tarentola mauritanica* and *Hemidactylus frenatus* respectively. The stellate arrangement of dense bodies, seen in cross section of the midpiece, in *T. mauritanica* closely resembles that in *H. binoei*. A stopper like electron dense perforatorial plate, seen in *H. binoei*, is illustrated for *T. mauritanica* and, less clearly, *L. picturatus*. A micrograph of the midpiece of *Sphaerodactylus cinereus* [46] shows a longitudinal series of four columnar mitochondria on each side (said to total 20 mitochondria for the midpiece) alternating with dense bodies, of comparable length, and the small annulus. In the absence of transverse sections it is difficult to compare this arrangement with that of other gekkonids but the columnar form of the mitochondria is reminiscent of that in *Heteronotia*.

Pygopodidae. The spermatozoa of *Lialis burtonis* (Fig. 8) are again like those of scincids in

Source : MNHN, Paris

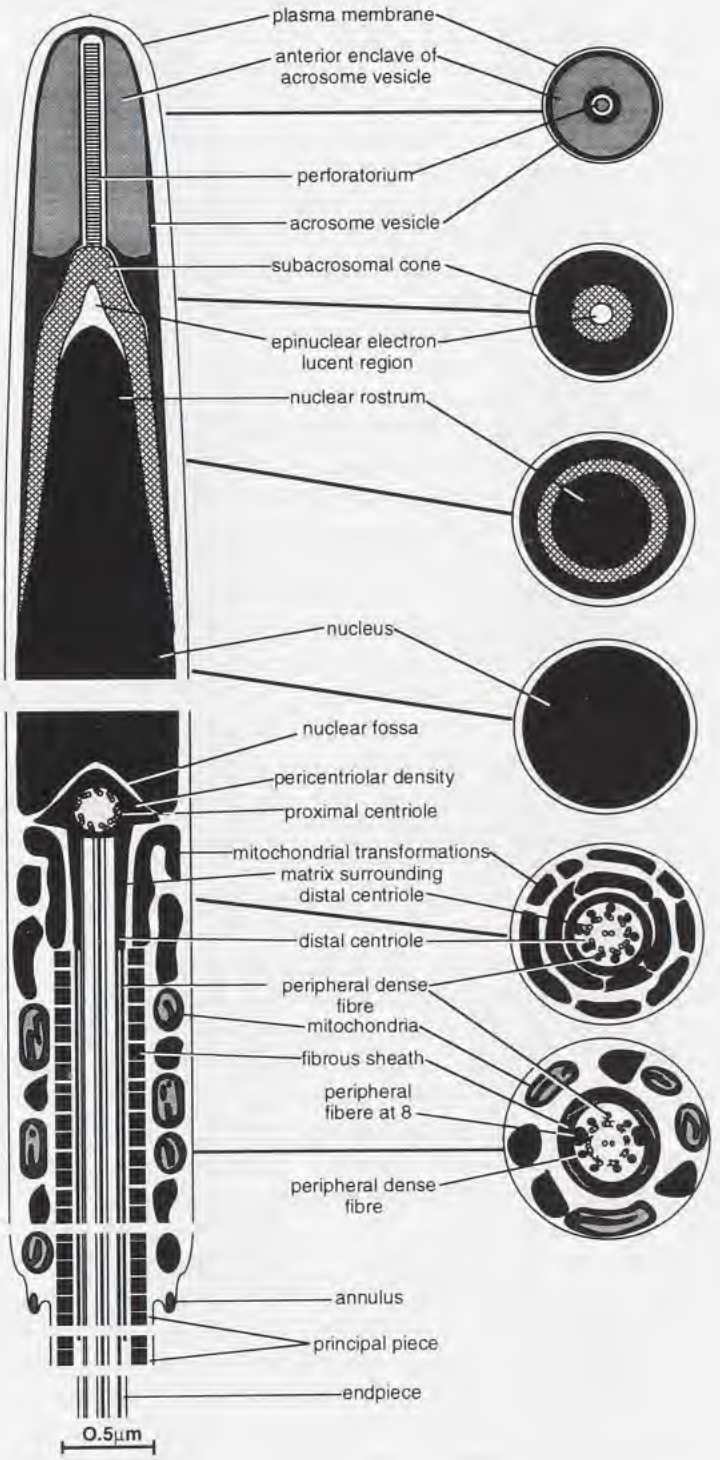


FIG. 8. — *Lialis burtonis* (Pygopodidae). A diagrammatic representation of the spermatozoon, in longitudinal and corresponding transverse sections. After [37].

Source : MNHN, Paris

their chief features, as was noted by HARDING *et al.* [25] in their preliminary report for the pygopods *Aprasia repens*, *Delma tincta*, *L. burtonis* and *Pygopus lepidopus*. However, in *L. burtonis* the acrosome is fore-shortened and apically domed and the perforatorium extends virtually to its tip; nuclear shoulders are absent; the mitochondria are small subspheroidal structures, four or five in a transverse section, and appear very numerous in longitudinal single file, alternating singly or in groups with one or more dense bodies, with evidence that they are sinuous; dense bodies also form an interrupted collar around the distal centriole [37]. The *L. burtonis* sperm shares a suite of apparently apomorphic character states of varying distinctiveness with Eugongylus-group skinks (*Cryptoblepharus virgatus*, *Carlia pectoralis* and *Lampropholis delicata*). They are as follows [37]. The perforatorium is square-ended rather than pointed. The midpiece is elongate, that in the Eugongylus-group species being less elongate but strikingly longer than that of sphenomorphs or other investigated squamates with the exception of the very long mitochondrial sheath of snake sperm [33, 45]. However, the mitochondria in *L. burtonis*, though forming a single layer as in snake sperm, are not as narrow. They are not irregularly interspersed with the dense bodies as they are in the Eugongylus-group skinks and are not as elongate as the mitochondria of the Eugongylus-group and snakes. Nevertheless, an elongated zigzag configuration much as in snake sperm is illustrated in a superficial longitudinal section of the midpiece of *Aprasia repens* by HARDING *et al.* [25] and these authors have noted a further striking similarity to snakes in the existence of a multilaminar membrane around the midpiece in their material of *L. burtonis*, and around the flagellum in *Delma tincta*. These multilaminar membranes had previously been considered unique to snake sperm [21, 33]. The co-occurrence of elongate, tubular, zigzagged mitochondria and an, albeit transient, multilaminar cell membrane in the sperm of pygopodids and snakes is here considered to warrant serious consideration that these two groups of legless squamates share a common origin despite lack of a direct sister-group relationship in the cladistic analysis (Figs 10, 11).

Serpentes. AUSTIN [3] gave a detailed account of the fine structure of the sperm tail in *Lampropeltis getulus*, *Coluber constrictor*, *Drymarchon corais*, *Crotalus adamanteus*, *Micrurus fulvius* and *Constrictor* sp., BOISSON & MATTEI [5, 6] described spermiogenesis in *Python sebae*. HAMILTON & FAWCETT [24] gave details of the neck and midpiece in *Lampropeltis getulus* and *Constrictor constrictor*, SAITA *et al.* [49] described spermiogenesis in *Coluber viridiflavius*, PHILLIPS & ASA [46] described the formation of the midpiece with reference to the behaviour of the annulus in *Masticophis flagellum flagellum* and AFZELIUS [1] described occlusion of microtubules in *Liophis miliaris*. Nevertheless, only FURIERI [20, 21] had given an account of the ultrastructure of the entire spermatozoon (giving in the latter work a general account for four species of Colubridae, *Coluber viridiflavius viridiflavius*, *Natrix tesselata tesselata*, *N. natrix*, and *Coronella austriaca*, and one species of Viperidae, *Vipera aspis aspis*) until the spermatozoon of *Nerodia sipedon* was described by JAMIESON & KOEHLER [33]. OLIVER *et al.* [45] describe the sperm of *Boiga irregularis*, *Stegonotus cucullatus* (Colubridae), *O. microlepidotus* (Elapidae), and *Aspidites melanocephalus* (Boidae).

Snake sperm (Fig. 9) present features [3, 20, 21, 24, 33, 45, 46] common to squamate sperm: they are filiform; the acrosome vesicle is in the form of a hollow, concentrically zoned cone which basally overlies a subacrosomal cone which invests the tapered anterior end of the nucleus; the perforatorium is a slender rod extending anteriorly from the subacrosomal material; the midpiece contains dense bodies (mitochondrial transformations) in addition to the mitochondria; the fibrous sheath surrounding the axoneme extends anteriorly into the midpiece (squamate autapomorphy); nine peripheral dense fibres accompany the triplets of the distal centriole and the doublets of the axoneme in the midpiece; the fibres adjacent to doublets 3 and 8 are enlarged, each as a double structure associated with the fibrous sheath; the endpiece lacks peripheral fibres and the fibrous sheath. A poorly developed "stopper-like" perforatorial base plate

Source : MNHN, Paris

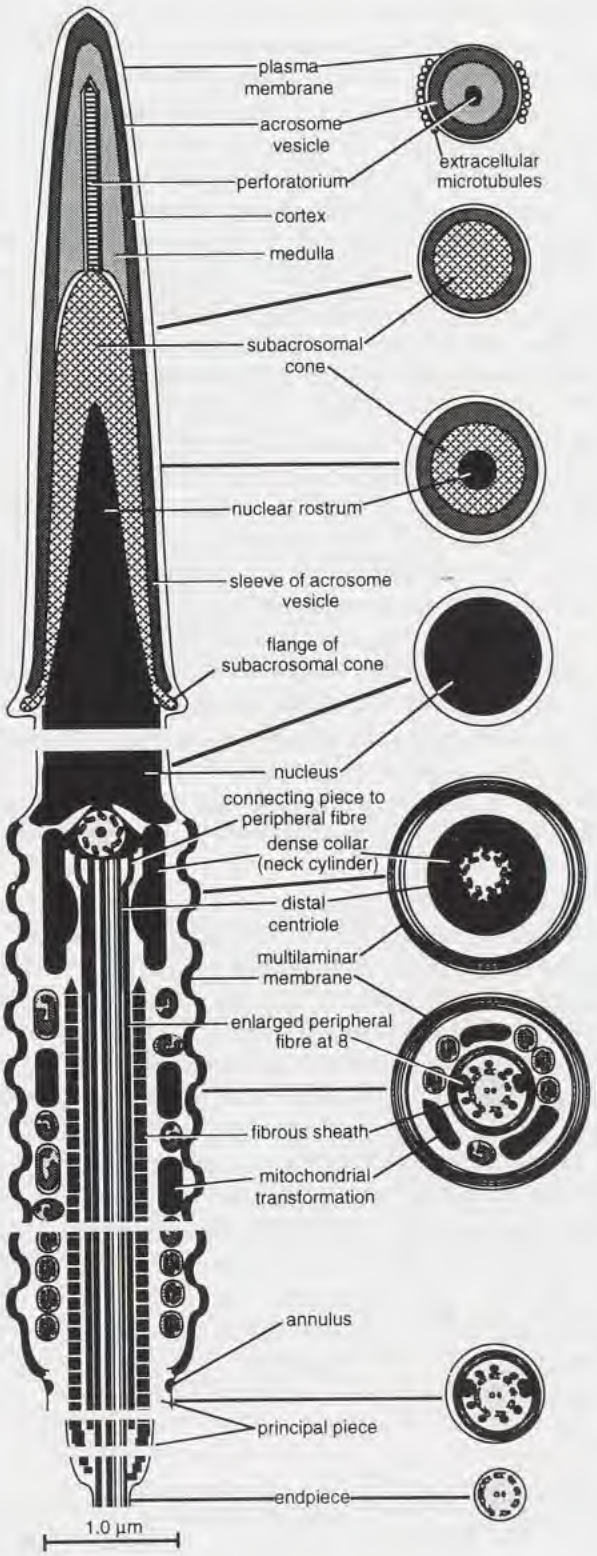


FIG. 9. — A diagrammatic representation of the spermatozoon of the Serpentes, in longitudinal and corresponding transverse sections. After [45].

Source : MNHN, Paris

in the colubrid *Nerodia sipedon*, unknown in other snakes, is presumably homoplasic with that of gekkonids. An electron-lucent space caps the nuclear point in the snakes *Boiga irregularis* and *Stegonotus cucullatus* as in some other squamate orders [33, 45] but is poorly developed.

Less widespread is a suite of apparently apomorphic characters states of snake sperm [33, 45] shared with Eugongylus-group skinks (*Cryptoblepharus virgatus*, *Carlia pectoralis* and *Lampropholis delicata*) and pygopodids. The shared states are as follows. The midpiece is greatly elongated in snakes (mitochondrial sheath of FURIERI [21]), that in the Eugongylus-group species, and pygopodids but also in gekkonids, being less elongate but strikingly longer than that of sphenomorph skinks or other investigated squamates. The mitochondria in at least some pygopodids [25] form zigzagged tubes as in snakes. They are not irregularly interspersed with the dense bodies as they are in the Eugongylus-group skinks. A multilaminar membrane is seen in some pygopods as in snakes (see above). JAMIESON *et al.* [37] stated that these similarities of snake, pygopodid and Eugongylus-group sperm warranted further investigation with a view to determining the degree of homoplasy as against synapomorphy. Individually these characters appear in some other squamates. Thus we have seen that extracellular tubules occur in immature teiid sperm and that multilaminar membranes invest the acrosome in immature tropidurid sperm. Possibly both types of structure are a normal feature of developing squamate sperm. Two apomorphies of snakes and pygopodids, the multilaminar membrane and the tubular zigzagged mitochondria are striking, and intuitively were considered synapomorphic resemblances. However, multilaminar membranes computed as basal not only to these two groups but also to Eugongylus-group skinks, in which they were computed as lost, and sinuous mitochondria parsimoniously (but not, perhaps, plausibly) computed as a basic squamate feature.

The evidence, albeit uncertain, for transformation of extracellular tubules into multilaminar membranes in *Boiga irregularis* possibly represents a stage in production of the membranes. Conversion of microtubules into membrane-like laminate appendages of testicular sperm is known in the Lepidoptera (see review in Jamieson [30]). It has been suggested that the multiple layers of membranes provide a source of endogenous phospholipid that could be utilized as a source of energy for motility [24].

The dense collar (termed the neck cylinder by AUSTIN [3]) is considered to be homologous with the intermitochondrial dense bodies [24, 45] which in turn have been shown in skinks (e.g. *Cryptoblepharus virgatus*) to be derived from mitochondria [37]. The dense element in the central axis of the proximal centriole, seen in *Aspidites*, is also illustrated and described for *Lampropeltis getulus* [24] and is stated to be regularly found in mammalian sperm. They [24] also observe the presence, unknown in other vertebrates, of extracellular microtubules, observed here in at least *Aspidites melanocephalus* and *Boiga irregularis*. With regard to the axoneme, they remind us that enlargement and prolongation of the peripheral fibres 3 and 8 in snakes (as, we note, in all squamates) is the reverse of the situation in the mammalian sperm tail where these are the smallest fibres and terminate first. The density associated with one of the two central singlets [3, 24], thought to be unique to snake sperm, has since been demonstrated for the skinks *Nangura spinosa* [34], *Tiliqua scincoides* and less certainly *Carlia pectoralis* [35], the gekko *Heteronotia binoei* [37] and the teiid *Cnemidophorus sexlineatus* [42] and may well be more widely demonstrated in squamates when favourable sections of the centriole are obtained. Dense material filling the A subtubules in the posterior portion of the axoneme has been demonstrated for doublets 1, 2, 5, 6 or 1, 2, 5, 6 and 7 in *Liophis miliaris* [1].

Parsimony analysis

A posteriori to intuitive consideration of comparative spermatozoal ultrastructure, with some inferred relationships, which has been detailed above, preliminary parsimony analyses have been

Source : MNHN, Paris

TABLE 1. — Ultrastructural characters of squamate sperm used in parsimony analysis. Character states numbered in parentheses are not transformation series as polarity was determined by use of an outgroup. The first state is nevertheless considered plesiomorphic.

Character	States
1 Acrosome TS	(0) circular (1) depressed,
2 Perforatorial base plate	(0) absent or indistinct (1) knoblike (2) stopperlike,
3 Perforatorial tip	(0) pointed (1) square ended,
4 Perforatoria, number	(0) two or more (1) one,
5 Epinuclear lucent zone	(0) absent (1) poorly developed (2) well developed,
6 Midpiece	(0) short (1) moderately long (2) very long,
7 Mitochondria in TS	(0) regular circlet (1) not regular (2) intermediate,
8 Mitochondria, shape	(0) rounded (1) columnar (2) sinuous tubes (3) 'intermediate rounded-columnar',
9 Dense bodies	(0) intramitochondrial (1) regular rings (2) scattered (3) linear series (4) stellate spiral (5) 2 groups,
10 Dense bodies, if regular	(0) not applicable (1) solid (2) granular (3) single file granules,
11 Cristae	(0) concentric (1) linear,
12 Nuclear shoulders	(0) sharp (1) rounded (2) absent,
13 Nuclear shape	(0) elongate (1) stout,
14 Endonuclear canal	(0) present (1) absent,
15 Fibres 3 and 8	(0) enlarged (1) grossly enlarged anteriorly,
16 Multilaminar membranes	(0) absent (1) present,
17 Fibrous sheath	(0) not in midpiece (1) in midpiece

performed using the PAUP program. The characters employed are those listed in Table 1 and the states of these characters are given in Table 2 which provides a summary of ultrastructure discussed in this account.

A branch and bound search was performed for the total data matrix (Table 2). The following options were applied: addition sequence: furthest; 1 tree held at each step during stepwise addition; MULPARS option in effect; steepest descent option not in effect; initial upper bound: unknown (compute via stepwise); branches having maximum length zero collapsed to yield polytomies; no topological constraints; trees unrooted; multi-state taxa interpreted as polymorphism; character-state optimization accelerated transformation (ACCTRAN). All characters were treated as unordered.

The branch and bound analysis produced 240 most parsimonious trees from which a strict (Fig. 11) and a 50% majority rule (Fig. 10) consensus tree was computed. These had the following characteristics in both analyses: tree length = 43; consistency index = 0.767; homoplasy index = 0.302; retention index = 0.839; rescaled consistency index = 0.644. Character state changes are included in Fig. 10.

If the search was made with multi-state taxa scored as uncertainty (to allow for the equivocal condition of the mitochondrial derivatives, character 9, in *Pogona*) an identical number of trees and topology were obtained but the tree was three steps shorter, with consistency index = 0.750; homoplasy index = 0.250; retention index = 0.839; rescaled consistency index = 0.629.

It must be stressed that the strict consensus tree (Fig. 11), agreed closely with the majority rule tree, differing only in placing *Cnemidophorus* on the same level as the remainder of its clade and *Lialis* on the same level as the *Carlia* and snake clades. These two polytomies correspond with the only percentages below 100% on the majority rule tree.

Source : MNHN, Paris

TABLE 2. — Comparative ultrastructure of spermatozoa of Squamata, *Sphenodon punctatus* and Chelonia. (Input data matrix).

Taxon	Characters 11111111 12345678901234567	Reference
Chelonia	00000000000000000	[26, 27]
Sphenodon punctatus	00000000000000000	[26, 27, 32]
Ctenotus robustus	10011001111101001	[37]
Chalcides ocellatus	1001?001131101001	[21]
Lacertidae	10011001501101001	[7, 11, 21]
Cnemidophorus sexlineatus	10012001111101001	[41, 42]
Tiliqua scincoides	10010001111101001	[35]
Carlia pectoralis	01112112201011101	[35]
Lampropholis delicata	01112112201011101	[37]
Heteronotia binoei	02010101401101001	[37]
Lygodactylus picturatus	02012101401101001	[21]
Lialis burtonis	0?112112301211011	[25, 37]
Pogona barbata	11012023111101001 3	[45]
Varanus gouldii	11012001121101001	[45]
Colubridae	00011212301101011	Serpentes:
Elapidae *(Oxyuranus)*	0001?212301101011	[3, 20, 21, 24, 33, 45, 46]
Boidae *(Aspidites)*	0001?212301101011	"
Iguanidae	00012002111001001 2 1	[22, 48]
Bradypodion karroicum	10012102201101001	This study

Discussion of the phylograms

The majority rule phylogram (Fig. 10) indicates those similarities of spermatozoal ultrastructure within the Squamata, and relative to *Sphenodon* and the Chelonia, which are synapomorphic for the respective nodes. These similarities, as in all spermiocladistic studies, must reflect similar fertilization biology but also phylogenetic, i.e. genetic, constraints. Spermatozoal morphology is here considered sufficiently conservative to reflect at least close phylogenetic relationship as it can be expected to be subject to stabilizing selection [22]. The evidence of many other studies (see, for instance, [26, 27, 31, 32] and references in [34]) shows that, although caution is required in their interpretation, such spermiocladistic phylograms hold significant phylogenetic information. Bearing in mind the necessity for caution and also the pressing need for inclusion of reliable data from a much larger sample of taxa, and confirmation of some data from the present sample, many of the following conclusions drawn from the phylogram can be regarded only as heuristic. Findings which both intuitively and by computation are unequivocal are stressed. Because of limitations of space, references to the literature will chiefly be confined to the excellent review of squamate classification by RIEPPEL [47].

Squamata. The Squamata, which are monophyletic in all most parsimonious trees in the analysis, are apomorphic relative to the Sphenodontida and Chelonia in the following features which are synapomorphies defining the Squamata s. strict. *Sphenodon* and the Chelonia are equally plesiomorphic and indistinguishable on the characters employed and therefore reference will chiefly be made to *Sphenodon*. It should be stressed, however, that *Sphenodon* is not here

Source : MNHN, Paris

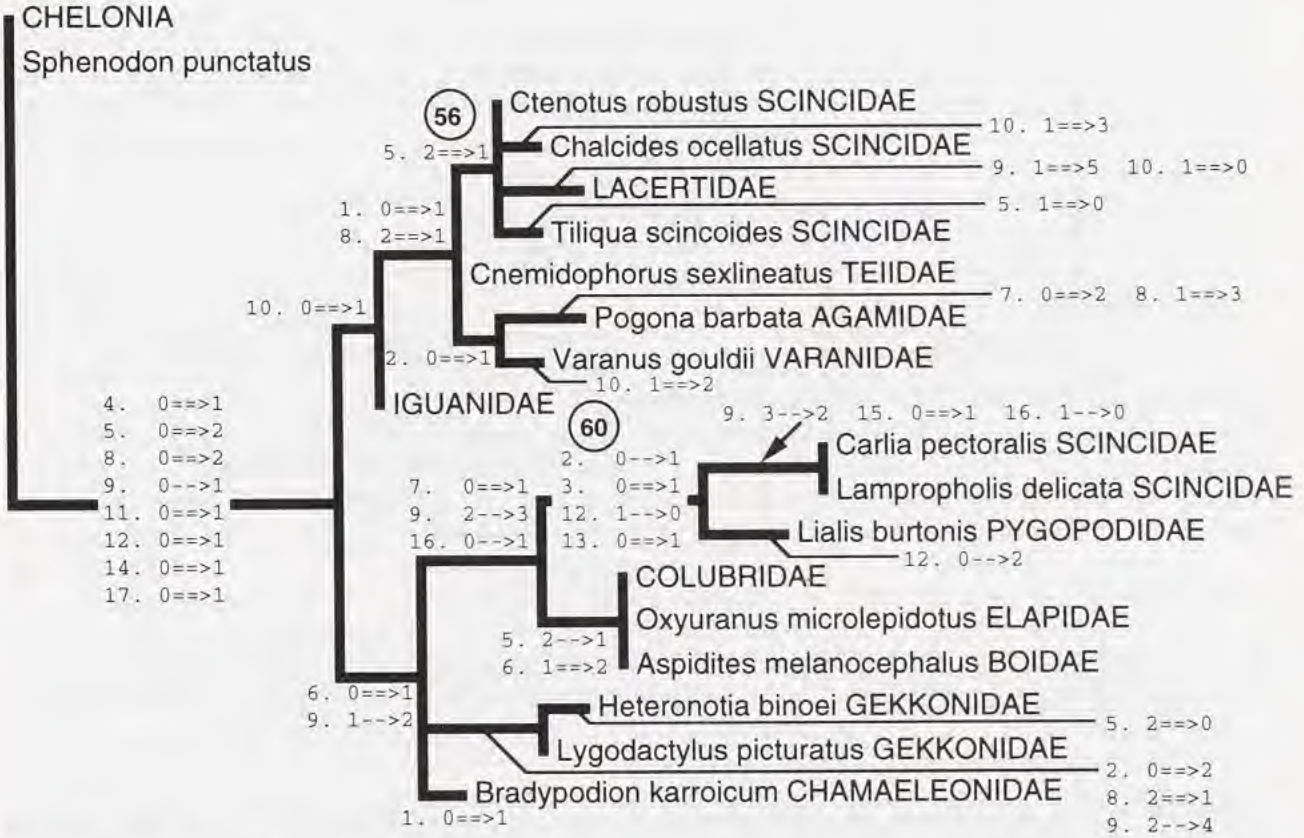


FIG. 10. — Branch and Bound 50 % majority rule consensus phylogram for 240 equally most parsimonious trees, for character states of chelonian, sphenodontid and squamate sperm ultrastructure listed in Tables 1 and 2. Settings as in text. Tree length = 43; consistency index = 0.767; homoplasy index = 0.302; retention index = 0.839; rescaled consistency index = 0.644. Numbers in circles are the percentage of trees supporting the node to which they attach. All other nodes were supported in 100 % of the trees.

regarded as the immediate sister-group of the Squamata *sensu strictu* but as a very basal amniote in a sphenodontidan lineage which possible predates emergence of the Crocodilia, Aves, and, it appears, the Mammalia [26, 32].

The squamate synapomorphies follow: 1. Possession of a single perforatorium in place of the two or three of Sphenodontida; 2. Loss of the endonuclear canal. The wholly epinuclear rather than partly endonuclear perforatorium is a notable difference of the Squamata from Chelonia, Sphenodontida, Crocodilia, non-passerine birds, and also basal lissamphibians (urodeles and *Ascaphus*). 3. Presence of the (well developed) epinuclear electron-lucent region. 4. Presence of sinuous mitochondria (possibly an artefactual parsimony resolution as a columnar form is intuitively preferred). 5. linear cristae, not the subspherical mitochondria, with concentric cristae of *Sphenodon*. 6. Dense bodies (mitochondrial transformations) intermitochondrial, in contrast with the intramitochondrial dense body in *Sphenodon*, and (equivocally) forming regular rings. Although the intermitochondrial position may well be basic to all squamates, one might query that the arrangement in regular rings is in their ground plan. 7. The fibrous sheath extends into the midpiece. This is a most significant and convincing synapomorphy and autapomorphy of the

Source : MNHN, Paris

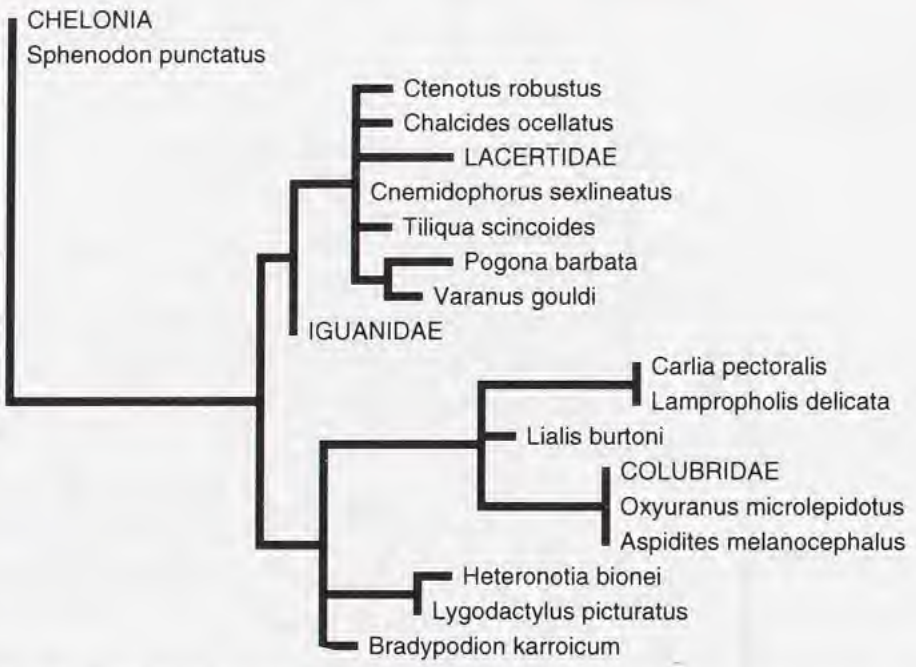


FIG. 11. — Branch and Bound 50 % strict consensus phylogram for 240 equally most parsimonious trees, for character states of chelonian, sphenodontid and squamate sperm ultrastructure listed in Tables 1 and 2. Settings, length and indices as in Fig. 10.

Squamata and is not seen in the Sphenodontida [26, 32]. 8. Development of rounded nuclear shoulders is basic to squamates. In addition, though not included in the parsimony analysis, squamates are diagnosed by the paracrystalline substructure of the subacrosomal cone.

These squamate spermatozoal autapomorphies constitute a striking endorsement of the "highly corroborated" monophyly of the Squamata for which evidence is reviewed by RIEPPEL [47].

'Sauria'. The Sauria is the third group of the Squamata, the other two being the Amphisbaenia and the Serpentes. Whereas the latter two groups appear to be monophyletic, RIEPPEL [47] stated that monophyly of the Sauria could not be established and that the name should be dropped or, in effect, broadened to equate with Squamata. If the evidence of the spermatozoal phylograms (Figs 10, 11) be accepted, the view that Sauria (in the strict sense of 'lizards' rather than squamates in general) are not monophyletic is emphatically endorsed as some saurians (invariably Eugongylus-group skinks and pygopodids, and, less closely, gekkonids and the chamaeleonid) appear more closely related to snakes than they are to other 'lizards'. Thus, Sauria is only monophyletic when it is, indeed, equated with Squamata and it includes lizards and snakes. The position of the amphisbaenians remains to be investigated when spermatozoal samples become available.

Scincomorpha. Monophyly of the Scincomorpha, though well supported by somatic morphology, has been considered to deserve further critical study [47]. The group is generally held to include the Xantusioidea, Lacertoidea (Lacertidae, Teiidae and Gymnophthalmidae) and the Scincoidea (Scincidae and Cordylidae). Spermatologically (Figs 10, 11) the Scincomorpha is diphyletic or possibly polyphyletic. This is due to the fact that the Scincoidea, represented in the parsimony analysis by the Scincidae only, are themselves at least diphyletic, notably because the Eugongylus-group skinks associate with the Serpentes, Pygopodidae, Chamaeleonidae and Gekkonidae

Source : MNHN, Paris

RIEPPEL (e.g. [47]) has repeatedly questioned the monophyly of the Scincidae and his doubts are underlined spermatologically. Thus the Eugongylus-group skinks (*Carlia* and *Lampropholis*) constitute the sister-group of the Pygopodidae; and the pygopodid-Eugongylus-clade is in turn the sister-group of the Serpentes in the majority rule tree (Fig. 10). However, the pygopodid forms a trichotomy with the Eugongylus-group and Serpentes clades in the strict consensus tree (Fig. 11).

The apparent synapomorphies joining the *Carlia* through Serpentes clades are weak. They are loss of the regular mitochondrial circlet, and, equivocally, development of linear dense bodies and of multilaminar membranes. Of these, the last two conditions are not seen in *Carlia* and *Lampropholis*. These two genera are linked to the pygopodid by four apomorphies. Two of these, the square-ended perforatorium and alteration of the nucleus to a stout form appear strong synapomorphies, while two are equivocal, acquisition of a knob-like basal plate (homoplasic with *Pogona* and *Varanus*) and development of sharp nuclear shoulders.

The severance of the Eugongylus-group from other skinks merits further investigation using non-spermatozoal characters. The spermatozoal synapomorphies defining the Eugongylus-group are striking. They are the characteristic development of scattered dense bodies, forming more than one layer around the axoneme; the fact that coarse fibres 3 and 8 are grossly enlarged anteriorly in addition to the usual enlargement in squamates; and, equivocally, reversion to no multilaminar cell membranes.

The position of the Lacertidae and Teiidae is poorly resolved (Fig. 10), and further data on these families are required. Linkage of the Lacertidae and Teiidae in the Lacertoidea [47] is not upheld. A teiid relationship to the Iguanidae (references in [47]) is also not supported. In the strict consensus tree (Fig. 11), lacertids, the teiid *Cnemidophorus*, and the skinks *Ctenotus*, *Chalcides*, and *Tiliqua* form an unresolved polytomy with the *Pogona-Varanus* clade. However, in the 50% majority rule tree (Fig. 10), *Cnemidophorus* is less close to the other scincomorphs than they are to each other. Intuitive considerations had placed teiids near sphenomorph (*Ctenotus*) and egernid (*Tiliqua*) skinks [37] but an especially close relationship is not upheld in this analysis.

Lacertid sperm have not been examined by the author but the composite data for the family, while placing them with the other, non-Eugongylus-scincomorphs, do not at present ally them more closely to any scincid or with the teiid. Their synapomorphies are arrangement of dense bodies into two groups and, of uncertain validity, reversal to a poorly developed epinuclear electron-lucent region. As recorded in the comparative section above, the arrangement of dense bodies in lacertids appears to be unusually diverse but is never strongly similar to the *Ctenotus* condition.

Iguania. Traditionally these include the Iguanidae, Anolidae, Tropiduridae, Agamidae and Chamaeleonidae. The Iguania does not emerge as a monophyletic group in the present analysis (Figs 10, 11). *Pogona* groups not with an iguanian but with *Varanus* in a clade which otherwise consists of non-Eugongylus scincomorphs while iguanids form the plesiomorph sister-group of this clade. The plesiomorphic status of the Iguanidae relative to agamids (references in RIEPPEL [47]) is endorsed but here in a paraphyletic relationship.

The chamaeleon Bradypodion is severed from the Iguanidae and is basal in the gekkonid-snake-pygopodid-Eugongylus-group clade in the majority rule (Fig. 10) and strict (Fig. 11) consensus trees in the present analysis. It may be noted, however, that in terms of patristic distance, the nearest taxon to the chamaeleon is, nevertheless, the Iguanidae. On intuitive consideration (see comparative account above) the sperm of the chamaeleon appear closely similar to that of the agamid in many respects but the sinuous tubular mitochondria are an important difference from agamids and, although a resemblance to iguanids, are also seen in the Carlia-pygopodid-snake clade. Much further investigation of spermatozoa, and comparison with morphological characters, is necessary if iguanian relationships are to be elucidated.

The knob-like basal plate appears to be the sole synapomorphy of *Varanus* and *Pogona* but is homoplasic with that in *Carlia* and *Lampropholis*.

Source : MNHN, Paris

Anguimorpha. Anguimorphs are conventionally divided into the Anguioidea and the Varanoidea [47]. Anguioidea have yet to be examined for sperm ultrastructure. In the comparative study above, the similarity of the *Varanus* sperm to that of *Pogona* has been described. From the comparative study, varanid sperm resemble those of agamids, sphenomorph and egernid skinks, and teiids in the depressed acrosome and intermitochondrial rings; they further resemble agamids and also (homoplasically) Eugongylus-group skinks in possessing a knob-like perforatorial base plate. Intuitively, as in the analysis (Fig. 10), the varanid is part of a sphenomorph-egernid-agamid assemblage. It is noteworthy that although a close relationships of varanids with scincids does not appear to have been previously suggested, brain data have been considered to indicate that the Teiidae are most closely related to the Varanidae [43].

A very close relationship between varanids and snakes which has formerly been postulated (see references in [45, 47]) is not supported by spermatozoal ultrastructure. *Varanus* sperm differ from those of snakes in the short midpiece, columnar mitochondria in a regular circlet, dense intermitochondrial bodies forming regular rings and absence of multilaminar membranes. It is expected that varanid relationships will come nearer to resolution when more than one species of each family is represented. A unique varanid apomorphy is replacement of the solid condition of the intermitochondrial rings, also seen in *Ctenotus*, *Cnemidophorus*, *Tiliqua*, *Pogona* and iguanids, with a granular condition.

Thus, the relationships of varanids remain enigmatic despite the parsimony analyses but a close relationship to snakes is not upheld.

Gekkota. Monophyly of the Gekkota, comprising the two extant families Gekkonidae and Pygopodidae has been considered well corroborated (references in RIEPPEL [47]). However, the majority rule consensus tree (Fig. 10) represents the Pygopodidae, exemplified by *Lialis burtonis*, as the sister-taxon of the Eugongylus-group skinks (for synapomorphies see Scincomorpha above). In the strict consensus tree (Fig. 11), *Lialis* forms a trichotomy with these skinks and the snakes. In the parsimony analysis (Figs 10, 11) the gekkonids form a monophyletic clade which, however, has an unresolved relationship with the chamaeleon, at the base of the snake+pygopodid+Eugongylus-group clade. The only unequivocal synapomorphy for the composite clade, is development of a moderately long midpiece which becomes very long in snakes. No unique synapomorphies are apparent between the sperm of gekkonids and pygopods which would support the special relationship between these two families suggested by KLUGE [38, 39, 40] who from a cogent cladistic study of general morphology has concluded [39] that the Pygopodidae should be placed within the Gekkonidae. This departure from somatic morphological evidence requires further investigation from sperm of larger numbers of taxa.

A relatively primitive position of the Gekkonidae in the 'Sauria' proposed by some workers [47], as opposed to the Pygopodidae, is supported by both consensus phylograms. Gekkos have somewhat generalized scincid sperm but a stellate arrangement of dense bodies in the midpiece distinguishes at least some of them. *Heteronotia* and *Lygodactylus* are sister-taxa in 100% of the trees. Computed synapomorphies of gekkonids (*Heteronotia* and *Lygodactylus*) are the stopper-like perforatorial base, the columnar condition of the mitochondria, supposedly derived secondarily from a sinuous condition; and, ambiguously, the stellate mitochondrial derivatives which compute as arising from a scattered condition such as is seen in Eugongylus-group skinks.

Serpentes. The origin of snakes, on the grounds of somatic morphology, continues to be an enigma [47]. However, the spermiocladistic analysis indicates origin of snakes from an ancestor, which must have been skink-like, shared with Eugongylus-group skinks (*Carlia* and *Lampropholis*) and with pygopodids. The pygopodid has a sister-group relationship with the Eugongylus-group skinks in the majority rule tree (Fig. 10) but forms an unresolved trichotomy with these and snakes in the strict consensus tree (Fig. 11). At present this origin of snakes must remain merely an heuristic finding but spermatozoa do provide a serpent apomorphy (see also [33, 45]) shared only with the pygopodid, presence of multilaminar sperm cell-membranes. These

Source : MNHN, Paris

membranes appear much better developed in snake sperm which differ further, and uniquely, in the immense elongation of the midpiece as a mitochondrial sheath around most of the length of the axoneme. That the multilaminar membranes have been lost in Eugongylus-group skinks, as computed, may be questioned. If not, presence would be a snake-pygopod synapomorphy. Further snake apomorphies appear to be reduction of the epinuclear electron-lucent region and loss of a perforatorial base plate. Snake sperm also have a greater development of extracellular tubules (not coded) than is known in any other squamate.

With regard to groups not included in the analyses, tropidurid sperm are insufficiently known for determination of phylogenetic affinities but the three gyres of mitochondria are also a lacertid characteristic; supposed failure of the fibrous sheath to penetrate the midpiece (the squamate autapomorphy) requires confirmation.

Investigations of sperm of additional taxa are required if suggested relationships throughout the Squamata are to be adequately tested.

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Source : MNHN, Paris

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Source : MNHN, Paris

Source : MNHN, Paris

Ultrastructure of Spermatozoa of Australian Blindsnakes, *Ramphotyphlops* spp. (Typhlopidae, Squamata): First Observations on the Mature Spermatozoon of Scolecophidian Snakes

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ABSTRACT

The structure of mature, epididymidal spermatozoa of three species of Australian blindsnakes, *Ramphotyphlops* spp. (Typhlopidae, Squamata) was investigated by a combination of scanning and transmission electron microscopy. Special attention was paid to features of potential phylogenetic significance, in the hope that aspects of sperm morphology might shed light on the evolutionary affinities of this highly specialized and enigmatic group of reptiles. Spermatozoa of *Ramphotyphlops* spp. share many features in common with other squamates (lizards and snakes), but display some striking features not known in any other taxa. Special derived features shared by *Ramphotyphlops* spp. and other Squamata include: a paracrystalline subacrosomal cone; dense intermitochondrial "rings" or "plaques"; a relatively inconspicuous annulus; an anteriorly extensive fibrous sheath which penetrates the midpiece; and the absence of an endonuclear canal. Additional derived features which may be shared exclusively by *Ramphotyphlops* spp. and other snakes include: the great elongation of the midpiece; the forward prolongation of the fibrous sheath to the level of the neck; the presence of a distinct "neck cylinder"; the extremely elongate form of the mitochondria; and the presence of a multilayered midpiece plasma membrane. These cladistic interpretations lend support to the current taxonomic placement of Typhlopidae within Squamata, and specifically within Serpentes. Several highly distinctive features of *Ramphotyphlops* spermatozoa are identified, the most striking being the extreme length and regular zig-zagging arrangement of the mitochondria.

RÉSUMÉ

Ultrastructure des spermatozoïdes des serpents aveugles australiens, *Ramphotyphlops* spp. (Typhlopidae, Squamata): premières observations sur le spermatozoïde mûr des Serpents Scolecophidiens.

La structure des spermatozoïdes mûrs épididymaires de trois espèces de serpents aveugles australiens, *Ramphotyphlops* spp. (Typhlopidae; Squamata) a été étudiée en microscopie électronique à balayage et à transmission. Une attention particulière a été portée aux détails d'importance phylogénique potentielle, dans l'espoir que des caractéristiques de la morphologie du spermatozoïde puissent éclairer les affinités évolutives de ce groupe de Reptiles, très évolués et

HARDING, H. R., APLIN, K. P., & MAZUR, M., 1995. — Ultrastructure of spermatozoa of australian blindsnakes, *Ramphotyphlops* spp. (Typhlopidae, Squamata): first observations on the mature spermatozoon of scolecophidian snakes. *In*: JAMIESON, B. G. M., AUSIO, J., & JUSTINE, J.-L. (eds), Advances in Spermatozoal Phylogeny and Taxonomy. *Mém. Mus. natn. Hist. nat.*, **166** : 385-396. Paris ISBN : 2-85653-225-X.

Source : MNHN, Paris

énigmatiques. Les spermatozoïdes de *Ramphotyphlops* spp. possèdent de nombreux caractères en commun avec les autres Squamata (lézards et serpents), mais montrent quelques aspects originaux inconnus dans les autres taxons. Les caractères dérivés spéciaux partagés par *Ramphotyphlops* spp. et les autres Squamata comprennent: un cône subacrosomien paracristallin, des "anneaux" ou "plaques" denses intermitochondriaux, un annulus relativement peu apparent, une gaine fibreuse s'étendant vers l'avant et qui pénètre la pièce intermédiaire et l'absence de canal endonucléaire. Les caractères dérivés supplémentaires qui pourraient être communs seulement à *Ramphotyphlops* spp. et aux autres Serpents comprennent: le grand allongement de la pièce intermédiaire, le prolongement vers l'avant de la gaine fibreuse jusqu'au niveau du cou, la présence d'un "cylindre du cou" distinct, la forme extrêmement allongée de la mitochondrie et la présence d'une membrane plasmique à plusieurs couches dans la pièce intermédiaire. Ces interprétations cladistiques soutiennent le placement actuellement admis des Typhlopidae dans les Squamata, et spécifiquement dans les Serpentes. Quelques caractères très originaux du spermatozoïde de *Ramphotyphlops* sont identifiés, dont le plus frappant est la longueur extrême et la disposition régulièrement zigzaguante des mitochondries.

Despite the pioneering efforts of FURIERI [6] to promote interest in the ultrastructure of reptilian spermatozoa, many significant gaps in taxonomic coverage still mar our understanding of spermatozoal structure and evolution in this large and important group of vertebrates. This is particularly marked within the taxonomically diverse Order Squamata (collectively, the lizards and snakes), for which information is available for less than one third of the extant families.

In this paper we report the first observations on spermatozoa from members of the Family Typhlopidae, a group commonly known as blindsnakes. These highly specialized, fossorial snakes are represented on all continents, with major radiations in Australia, Africa and Central America. Together with members of at least two other families, Leptotyphlopidae and Anomelepidae, blindsnakes are generally included in the higher taxon Scolecophidia, as one of the two major groups of living snakes [4, 13]. However, some authors have suggested a possible closer relationship with certain groups of lizards, thereby challenging the monophyly of both Scolecophidia and Serpentes [9, 14]. Even more remarkably, one worker has questioned the placement of Typhlopidae within Squamata, based on peculiar features of the male reproductive tract [17].

Material examined in this study comes from three species of the Australasian blindsnake genus *Ramphotyphlops,* which includes approximately 50 species spread between Malaysia and southeastern Australia. This study forms one part of a wider comparative survey of spermatozoal morphology among Australian and Indonesian squamates, from which we hope not only to fill many of the major gaps in taxonomic coverage, but also to further knowledge of squamate phylogeny and classification.

MATERIAL AND METHODS

Spermatozoal samples were collected from three *Ramphotyphlops* species. The associated voucher specimens are lodged in the collection of the Western Australian Museum as follows: *Ramphotyphlops waitii* 114891 Waggrakine Pass, W. A. and 119239 Walyunga National Park, W. A.; *R. endoterus* 115000 38 km ENE Laverton; *R. australis* 115125 11 km ESE North Dandalup. Samples from all three species were processed for TEM and for *R. waitii* also for SEM.

Animals were euthanased with sodium pentabarbitol (Nembutal). Immediately following death, samples of testis and epididymides were removed, cut into small pieces and fixed for 30-60 minutes in Karnovsky's fixative with picric acid added. They were washed and stored in 0.1 M cacodylate buffer pending further treatment.

Samples for TEM were postfixed in 1% osmium tetroxide in cacodylate buffer, stained en bloc in 4% alcoholic uranium acetate, dehydrated in alcohol and acetone, and embedded in SPURR resin. Sections were stained in 4% alcoholic uranium acetate and lead citrate, and examined using a JEOL 100 TEM.

For SEM a drop of sperm released from the tissue was placed on a cover slip coated with platinum and then with poly-L-lysine. Samples were postfixed in osmium tetroxide in cacodylate buffer, dehydrated, critical point dried in CO_2, and examined using field emission, high resolution JEOL JSM-6000, at the accelerating voltage of 3 kV.

Measurements, which are of *R. waitii*, were taken from micrographs and using the 40/0.7 and 100/1.3 objective lenses of a Leitz Diaplan microscope with a video camera attached and connected to a MD30 Automated Image Analysis System (Leading Edge, Australia). However, owing to the extreme length of the spermatozoon and the difficulty of distinguishing the midpiece and principal piece junction in whole-sperm light microscopy, the measurements given in this paper should be regarded as approximate only.

Source : MNHN, Paris

OBSERVATIONS

The mature spermatozoon in all three species is clearly very elongate, with lengths up to 179 μm recorded for *R. waitii* under the image analyser.

Head

The sperm head is elongate and essentially cylindrical in form, but with a slight flexion just behind the base of the acrosomal cap. Measurements taken from micrographs indicate a total head length of 15 μm and a basal diameter of 0.6 μm. The nucleus consists of a basal cylindrical portion measuring 10 μm in length, and a tapered apical portion which projects 2.1 μm into the acrosomal cap (Fig. 1a). A distinct "nuclear shoulder" marks the transition from basal to apical regions (Fig. 1a, h). The nuclear fossa is broad and relatively shallow except in the very centre above the basal plate where it is more deeply excavated (Fig. 2c). The nucleus is evenly electron-dense and is not penetrated by an endonuclear canal.

The acrosomal complex forms a complete cap which sits atop the tapered portion of the nucleus (Fig. 1a); it measures 3.8-4.0 μm from nuclear shoulder to acrosomal tip. In common with other reptiles, the acrosome consists of three main components: an inner subacrosomal cone; an outer acrosomal sleeve; and a central, rod-like perforatorium (Fig. 1a). The perforatorium projects from the tip of the subacrosomal cone as a slender transversely striated rod (Fig. 1a, f-g). In *R. waitii* it measures 1 μm. As shown by comparison of Figs 1f and 1g the perforatorium varies in length between the species, being a much longer structure in *R. waitii* than in *R. endoterus*. Basally it is embedded within the subacrosomal cone (Fig. 1c, f-g). A narrow electron-lucent zone surrounds much of the striated perforatorium. In *R. waitii* and *R. australis* this vacuity is irregularly subdivided by a series of vertical folds which arise from the inner surface of the acrosomal sleeve (Fig. 1a, f). In *R. endoterus* the acrosomal vacuity is more prominent and is regularly partitioned by four vertical "vanes" of the acrosomal sleeve (Fig. 1b, g). The subacrosomal cone has a paracrystalline structure (Fig. 1a, e, g). It is surrounded by the acrosomal sleeve which is complex in form. Basally, where the sleeve overlies the subacrosomal cone, it consists of a single amorphous layer (Fig. 1a, d-e). Proximally, it is composed of two distinct units separated by a narrow, electron-dense band (Fig. 1a-b, f-g). A well-defined "trigger area" [5] covers the proximal tip of the sperm head (Fig. 1a, f-g).

The plasma membrane covering the acrosomal portion of the sperm head appears as a rather heavy and relatively "loose" membrane over most of the acrosome but it is attached to the nuclear membrane proximally around the margin of the "trigger area" and basally at the "post-acrosomal ring", an annulus-like structure which rests upon the nuclear shoulder (Fig. 1a, h). In common with various other groups of snakes and lizards [1, 7, 15], the plasma membrane of the sperm head is surrounded by a pallisade of closely-applied, longitudinally aligned microtubules (Fig. 1a, d-e, g-h).

Neck region

The neck region in *Ramphotyphlops* is exceptionally complex, comprising both proximal and distal centrioles; a basal plate which lines the nuclear fossa; a series of longitudinal columns which extend anteriorly from the dense outer fibres (peripheral fibres) of the midpiece; an electron dense body (referred to here as the "capitulum" in view of its similarity to the structure of that name in mammalian spermatozoa) which conforms to the shape of the basal plate anteriorly, surrounds the proximal centriole and joins to the longitudinal columns; and a "neck cylinder" which encloses the remaining neck elements and extends distally to surround the anterior extremity of the fibrous sheath.

The short proximal centriole (Fig. 2b, j) is set at an angle of approximately 80 degrees to the distal centriole and axoneme. It conforms to the usual pattern of nine sets of peripheral triplets and no central tubules. The central space is penetrated by a body of diffuse, electron dense material

Source : MNHN, Paris

representing a "centriolar pedicle" of the neck cylinder (Fig. 2b). This unusual structure is prominent in *R. endoterus*, in which the neck cylinder and its various processes are relatively electron dense (Fig. 2c, f-g). In contrast the neck cylinder in *R. waitii* and *R. australis* appears relatively electron lucent and granular (Fig. 2a-b, d-e). The proximal centriole as a whole is embedded within an irregular-shaped, electron dense body (the "capitulum") which closely parallels the form of the nuclear fossa (Fig. 2a-c, i). This structure forms the "ball" of a mortise and tenon joint between the sperm head and flagellum, and is therefore at least analogous with the capitulum that forms the anterior region of the "connecting piece" of mammalian sperm.

The basal plate forms the functional interface between the nuclear and flagellar portions of the spermatozoon. It appears to be of composite origin, including both nuclear membranes and membranes which envelope the midpiece and neck structures (Fig. 2 a-c). The space between the basal plate and the capitulum is occupied by an unbounded body of granular material, with a suggestion of radial structure in some micrographs (Fig. 2 a-b).

The distal centriole extends posteriorly from the proximal centriole (but parallel to the long axis of the flagellum) to approximately the region of the flagellum marked by the anterior extremity of the fibrous sheath (Fig. 2a, c, d-f), where it meets the axoneme. It possesses the usual peripheral triplets as well as a pair of central singlets (Fig. 2d). The dense outer fibres associated with each of the nine axonemal doublets continue forward into the neck where they meet the longitudinal columns. These nine columns diverge progressively from the centriolar triplets as they extend in the direction of the sperm head and at their anterior extremity they join the capitulum, forming a support for the proximal centriole and capitulum (Fig. 2a, c, h-j). Transverse sections through the distal centriole show a supernumerary dense column lying between one of the pair of central singlets and one of the centriolar triplets which, on the basis of presently incomplete information, is judged to be triplet 3 (Fig. 2 d-f). One of the longitudinal columns is very poorly developed relative to the others and generally is found close to its adjacent

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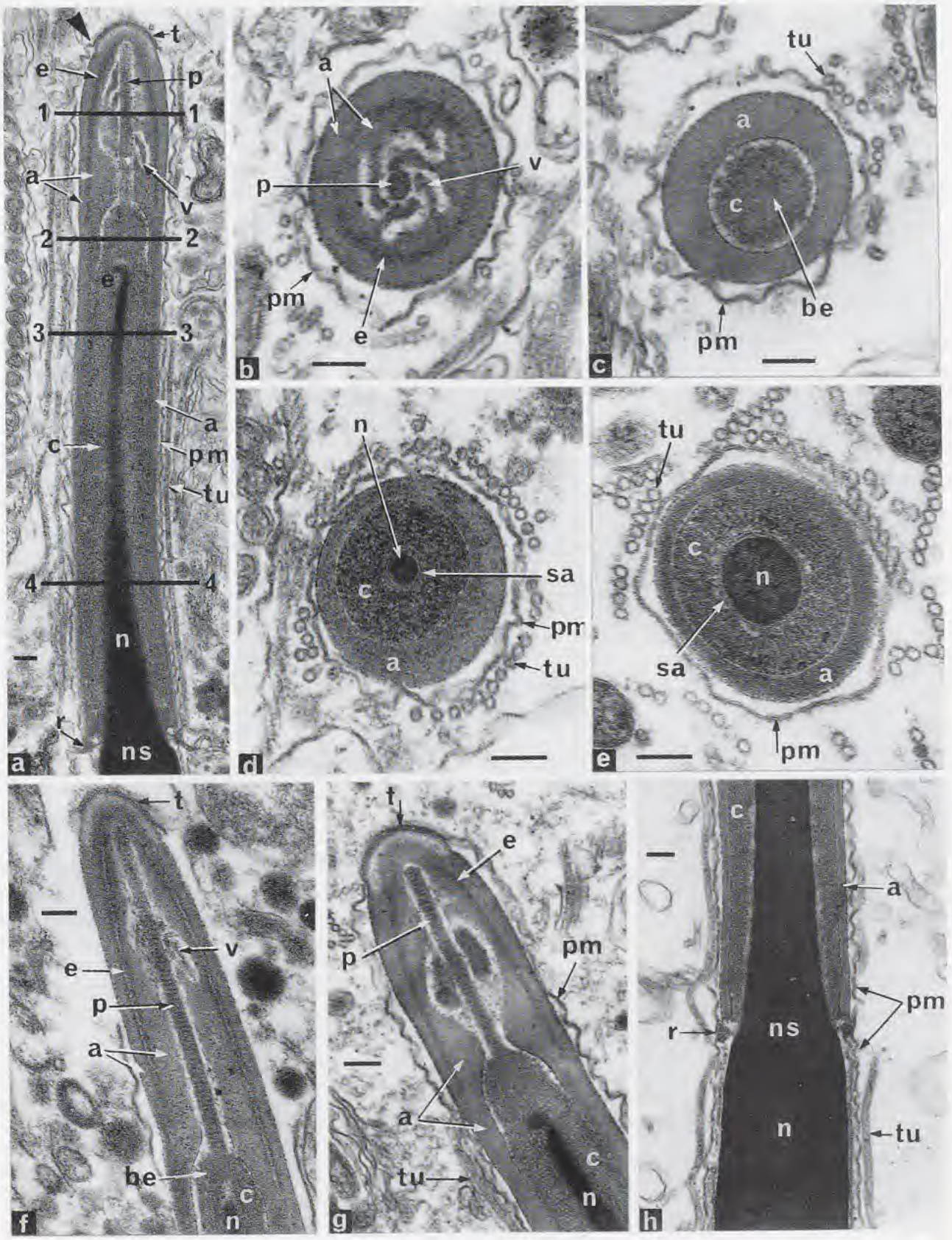
FIG. 1. — **a:** Longitudinal section through the acrosomal region of the nucleus in a *Ramphotyphlops waitii* spermatozoon. The arrow head marks the attachment of the plasma membrane and nuclear membranes at the trigger area and the post-acrosomal ring marks the basal attachment. The numbered lines indicate the approximate position of the transverse sections in Fig. 1b-e. **b:** Transverse section through the head of a *R. endoterus* spermatozoon in the position marked 1–1 in Fig. 1a. Note the regular arrangement of the vertical folds in the acrosomal vacuity. **c:** Transverse section through the head of a *R. endoterus* spermatozoon in the position marked 2–2 in Fig. 1a. A very slight variation in the appearance of the centre of the subacrosomal cone marks the basal portion of the perforatorium. **d:** Transverse section through the head of a *R. waitii* spermatozoon in the position marked 3–3 in Fig. 1a. Note the thin layer of subacrosomal material surrounding the nucleus. **e:** Transverse section through the head of a *R. waitii* spermatozoon in the position marked 4–4 in Fig. 1a. The paracrystalline structure of the subacrosomal cone is very evident. **f:** Longitudinal section through the anterior region of the acrosome in a *R. waitii* spermatozoon. Compare the length of the perforatorium with that of *R. endoterus* in Fig. 1g printed at approximately the same magnification. **g:** Transverse section through the anterior region of the acrosome in a *R. endoterus* spermatozoon. Note that the vertical folds enclosed within the acrosomal vacuity are more prominent and regular than in *R. waitii*. **h:** Longitudinal section through the head of a *R. waitii* spermatozoon in the region of the post-acrosomal ring. a-h, scale bar = 0.1 μm.

Abbreviations for all figures: a, acrosomal sleeve; an, annulus; b, basal plate; be, basal portion of perforatorium embedded in the subacrosomal cone; c, subacrosomal cone; ca, capitulum; cy, neck cylinder; d, distal centriole; df, dense outer fibres; e, electron dense band separating inner and outer layers of acrosomal sleeve; f, fibrous sheath; g, granular material surrounding fibrous sheath; i, intermitochondrial plaque; lc, longitudinal columns of the neck; m, mitochondria; n, nucleus; ns, nuclear shoulder; p, striated rod of the perforatorium; pd, centriolar pedicle; pm, plasma membrane; px, proximal centriole; r, post-acrosomal ring; s, supernumerary longitudinal column; t, trigger area; tr, centriolar triplets; tu, palisade of longitudinally orientated microtubules surrounding the spermatozoon; v, vertical folds within acrosomal vacuity; x, axoneme.

Source : MNHN, Paris



Source : MNHN, Paris

triplet (Fig. 2 d-e). The spatial relationship between this column and the supernumerary column is constant in the various transverse sections (Fig. 2 d-e) and, again on the basis of as yet incomplete information, this poorly developed column appears to be adjacent to triplet number 9.

The fibrous sheath, normally confined to the principal piece in tetrapod sperm, extends in *Ramphotyphlops* all the way forward to the level of the axoneme–distal centriole junction. In longitudinal sections the columns are seen to diverge radially from this point moving anteriorly (Fig. 2a, c, h-i).

It is not clear precisely where the columns join the dense outer fibres. In a region which must be just anterior to the commencement of the fibrous sheath (since the sheath is absent but the columns/fibres are not radially displaced from the axoneme doublets) the supernumerary fibre is still seen between the central pair and outer doublet 3 (?). Dense outer fibres 3 and 8 are particularly prominent and the fibres associated with all the doublets appear not only adjacent to the outer periphery of the circle of doublets, but also extend inwards, between and partially enclosing each doublet (Fig. 2f).

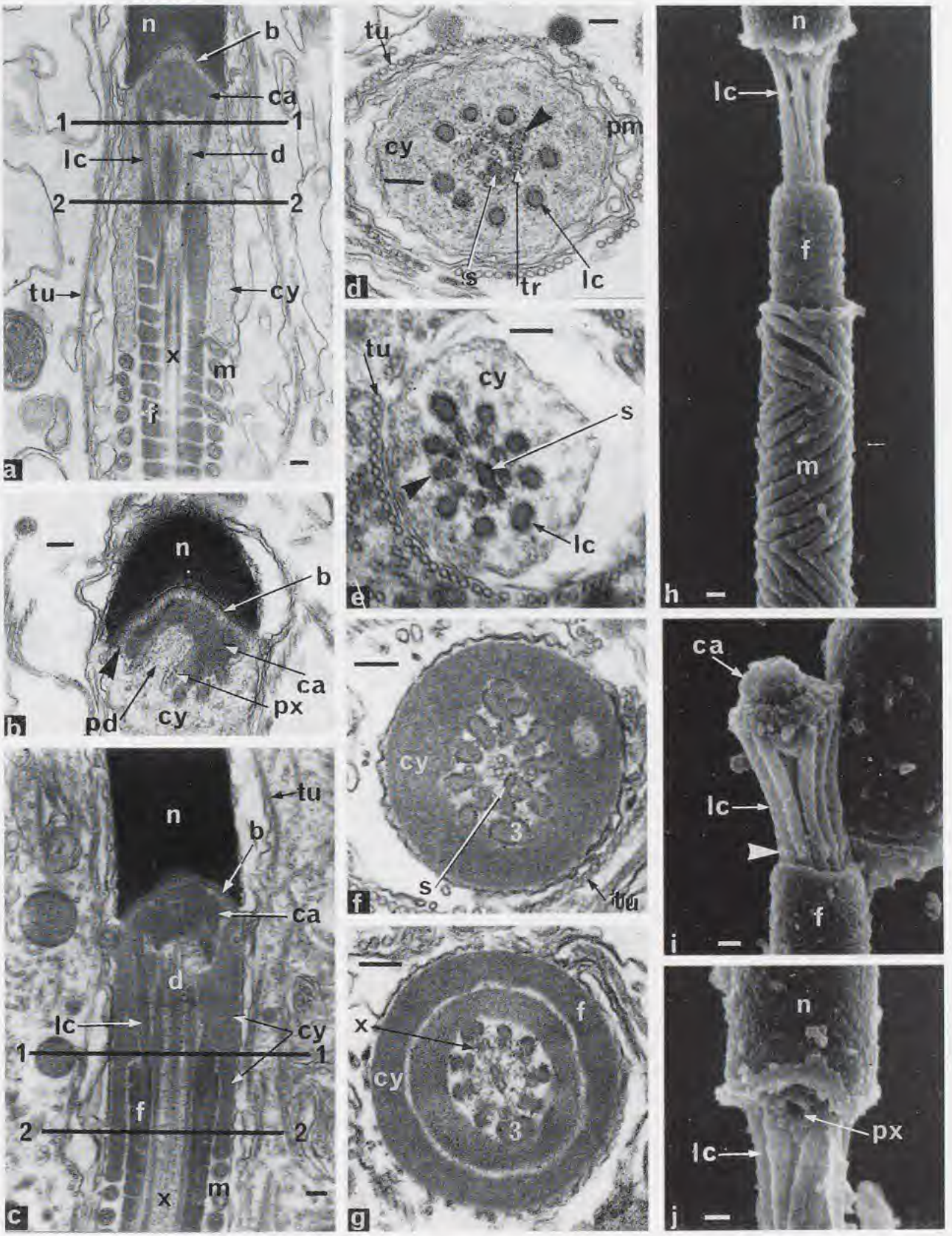
The neck cylinder in *R. waitii* and *R. australis* consists of a diffuse and somewhat uneven aggregation of granular material, bounded by a narrow membrane. Anteriorly, it surrounds the capitulum and the centriolar complex, contacting the nucleus around the periphery of the nuclear fossa, where it appears to be continuous with the basal plate (Fig. 2 a-b). Posteriorly, it extends well beyond the point of the distal centriole–axoneme union to enclose the anterior portion of the fibrous sheath (Fig. 2 a). In *R. endoterus* the neck cylinder is a more solid structure, being equivalent in electron density to the "capitulum" and the dense outer fibres (Fig. 2 c). However, its basic form and relations are identical to those described for *R. waitii*.

Midpiece

The midpiece is extremely elongate, with one complete section measured from a micrograph reaching 108 µm. Typical cross-sections are circular in shape, have a diameter of 0.5 µm and show nine mitochondrial sections; eight of which are round and one very elongate (Fig. 3 c).

FIG. 2. — **a:** Longitudinal section through the neck and anterior midpiece of a *Ramphotyphlops waitii* spermatozoon. The longitudinal columns diverge as they extend from the anteriormost portion of the fibrous sheath to the capitulum, which surrounds the proximal centriole. The numbered lines indicate the approximate position of the transverse sections in Fig. 2 d-e. **b:** Oblique longitudinal section through the nuclear fossa of a *R. waitii* spermatozoon. Note the radial striations in the granular material (arrowed) separating the basal plate and the capitulum and the centriolar pedicle of the neck cylinder. **c:** Longitudinal section through the neck and anterior midpiece of a *R. endoterus* spermatozoon. The numbered lines indicate the approximate positions of the transverse sections in Fig. 2 f-g. **d:** Transverse section through the distal centriole of a *R. waitii* spermatozoon in the approximate region marked 1–1 in Fig. 2a. Note the multiple plasma membranes, the poorly developed longitudinal column (arrowed), the supernumerary column, and the central pair surrounded by the centriolar triplets. **e:** Transverse section through the distal centriole of a *R. waitii* spermatozoon in the approximate region marked 2–2 in Fig. 2a. Note that the longitudinal columns are not as radially divergent as in Fig. 2d. The poorly developed column is arrowed. **f:** Transverse section through the neck region of a *R. endoterus* spermatozoon in the approximate region marked 1–1 in Fig. 2c. Note the difference in electron density of the neck cylinder compared with that in *R. waitii* (Fig. 2e). Note that the fibres extend between the doublets. Fibres 3 (labelled) and 8 are considerably larger than the rest but all 9 fibres are well developed. **g:** Transverse section through the neck region of a *R. endoterus* spermatozoon in the approximate region marked 2–2 in Fig. 2c. Note the supernumerary column is no longer visible and dense outer fibre (3?) (marked) is particulalry large. **h:** SEM view of the neck and anterior midpiece of a *R. waitii* spermatozoon. The plasma membrane and neck cylinder have been lost to reveal the underlying structures. **i:** SEM view of the neck of a *R. waitii* spermatozoon. The head and flagellum are separated to reveal the capitulum supported by the longitudinal columns. There appears to be a junction (arrowed) just anterior to the start of the fibrous sheath, which may be the junction between the longitudinal columns and the dense outer fibres. **j:** SEM view of the nucleus-neck junction in a *R. waitii* spermatozoon. The plasma membrane and neck cylinder have been lost to reveal the proximal centriole. a-j, scale bar = 0.1 µm.

Source : MNHN, Paris



Source : MNHN, Paris

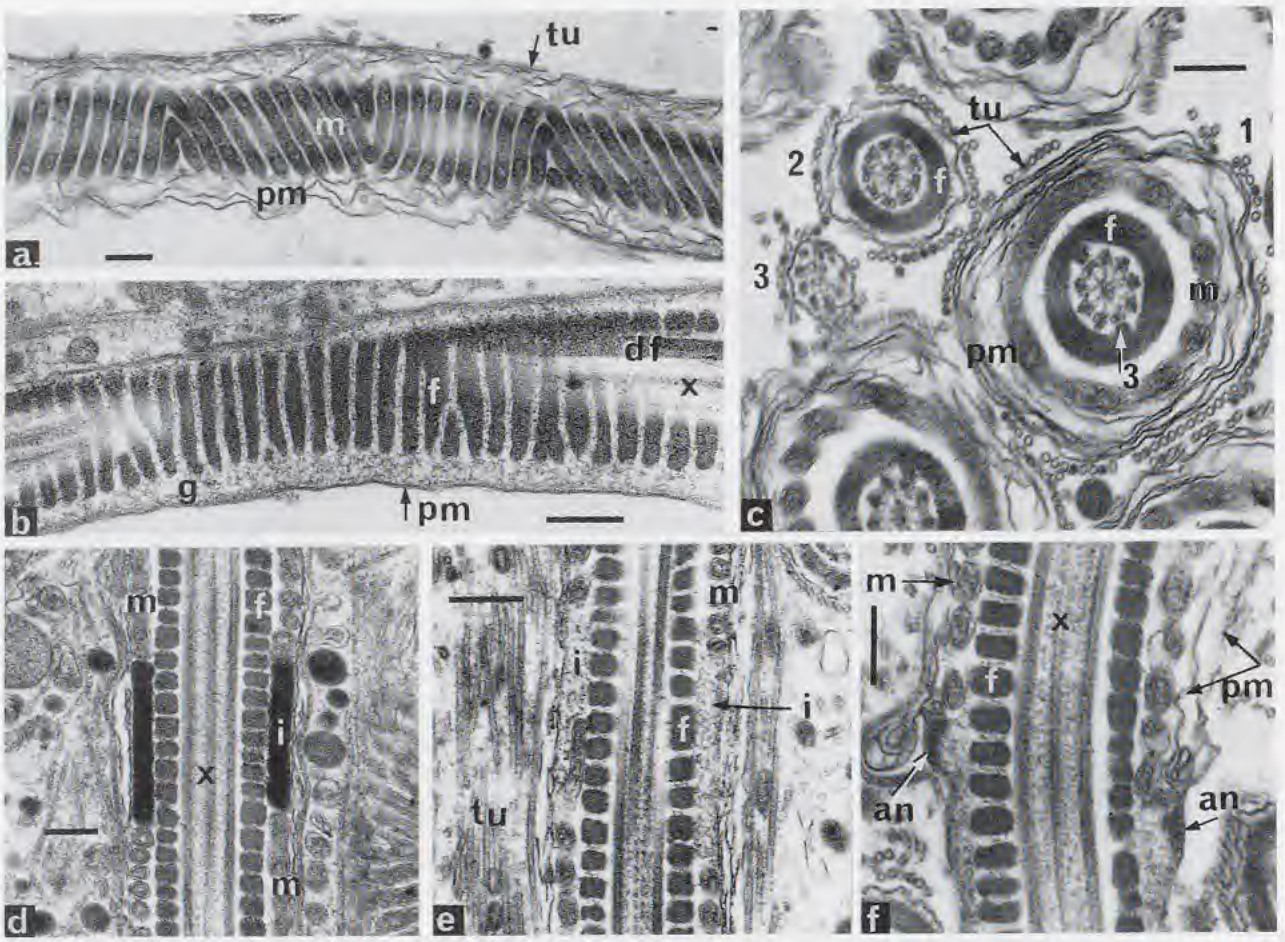


FIG. 3. — **a:** Grazing longitudinal section of the midpiece of a *Ramphotyphlops waitii* spermatozoon to show the ordered and unusual arrangement of the mitochondria. Note the multiple plasma membranes. **b:** Grazing longitudinal section of the fibrous sheath in the principal piece of a *R. waitii* spermatozoon. Note the single plasma membrane and the granular material surrounding the fibrous sheath. **c:** Three transverse sections of *R. waitii* spermatozoa to show the midpiece (labelled 1), the principal piece (2) and the endpiece (3). Note that in the midpiece the dense outer fibres 3 (numbered) and 8 are considerable larger than the others and are associated with the inner layer of the fibrous sheath, and that the fibres are absent in the principal piece section. Note also the multiple plasma membrane layers in the midpiece and the single layer in the principal and end pieces. **d:** Longitudinal section of part of the midpiece of a *R. endoterus* spermatozoon to show the intermitochondrial plaque. **e:** Longitudinal section of part of the midpiece of a *R. australis* spermatozoon to show the poorly developed intermitochondrial plaque. **f:** Longitudinal section of the annulus region of a *R. endoterus* spermatozoon. Note the multiple plasma membranes in the midpiece and single in the principal piece and the granular material in the principal piece. a-f, scale bar = 0.2 µm

Anteriorly, the mitochondrial sheath commences at the posterior extremity of the neck cylinder. As shown in Figs 2 a, 2 c and 2 h, it surrounds the fibrous sheath, which extends further anteriorly than the mitochondria.

Sections derived from anterior portions of the midpiece show 9 dense outer fibres, each closely applied to a peripheral axonemal doublet. As is common among non-mammalian amniote spermatozoa, fibres 3 and 8 are much larger than the others and make contact with the encircling fibrous sheath (Fig. 3 c). More distal sections show a progressive reduction, eventually leaving only fibres 3 and 8 as longitudinal "ribs" of the fibrous sheath.

Source : MNHN, Paris

The fibrous sheath as revealed in grazing sections has a predominantly transverse orientation but with irregular bifurcations and anastomoses (Fig. 3 b).

The mitochondria show a uniquely regular and complex arrangement which immediately distinguishes *Ramphotyphlops* spermatozoa from those of all other reptiles. As shown clearly in both grazing TEM sections (Fig. 3 a) and in SEM images (Fig. 2 h), the overall pattern consists of a series of stacked and alternating chevrons. SEM images of damaged sperm suggest that individual mitochondria are extremely elongate and narrow, with a diameter of around 0.1 μm and that they spiral helically around the midpiece for one complete revolution before turning sharply to repeat the pattern but with the opposite helical polarity (i.e. if clockwise, then anticlockwise). The exact length attained by any individual mitochondrion, as well as the number of flexion points per mitochondrion are presently unknown. Order is apparently maintained in this potential chaos by a very precise alignment of the points of flexion around a common circumferential point, thereby maintaining the pattern of regular, alternating chevrons. In some grazing sections of the midpiece the mitochondria show a more chaotic, intertwining and overlapping arrangement. It is not clear at this stage whether these sections are artefactual, resulting from cut spermatozoa, or whether they are a natural variant, possibly associated with posterior sections of the midpiece.

Transverse and longitudinal setions consistently show a broad translucent zone between the mitochondria and the underlying fibrous sheath (Fig. 3c, f). This apparent lack of contact between the two flagellar sheaths is surprising in view of the regular alignment of the mitochondria. Internally, the mitochondria usually show a single, circular crista enclosing granular material of variable density (Figs 2 a, 3 d).

Most transverse and longitudinal sections through the midpiece show that it is surrounded by multiple plasma membranes (generally between five and seven). The longitudinally orientated microtubules which so conspicuously surround the spermatozoal head are also evident in most sections of the midpiece (Fig. 3 a, c).

Intermitochondrial "rings" or "plaques" are clearly present in *R. endoterus*, where blocks of electron dense material are commonly observed in both tranverse and longitudinal sections, interspersed among otherwise typical mitochondria (Fig. 3 d). Available material suggests that both complete rings and discrete blocks are represented. Less compelling evidence for the presence of intermitochondrial rings is available for *R. australis* and *R. waitii*. In each species just one longitudinal section has shown apparently discrete, membrane-bound plaques of granular material interposed between typical mitochondria (Fig. 3 e). Notably, although the plaques are very definite structures in *R. endoterus*, they are not numerous in comparison to the pattern seen in sperm of many other reptile species [6].

Principal piece

The boundary between mid-piece and principal piece is marked by a small but distinct annulus. There is only a minor decrease in flagellar diameter between the midpiece and principal piece (Fig. 3f). The lack of abrupt narrowing of the flagellum posterior to the mitochondrial sheath is due to the presence of a diffuse granular material both external to, and intercalated between, the elements of the fibrous sheath of the principal piece (Fig. 3 f). Further posteriorly, this granular layer is absent, and the principal piece is correspondingly smaller in diameter.

The longitudinal fibres associated with the fibrous sheath, adjacent to axoneme doublets 3 and 8, extend an unknown distance into the principal piece. The absence of these fibres from most transverse sections of the principal piece (Fig. 3 c) suggests that they must terminate fairly close to the annulus.

A single plasma membrane is present throughout the length of the principal piece, as are the associated microtubules (Fig. 3 c). In sections of *R. endoterus* the multilayered membranes of the

Source : MNHN, Paris

midpiece are clearly seen to terminate at the annulus, posterior to which a single, heavier membrane is present (Fig. 3f).

The length of the principal piece could not be measured from available material. However, it must be at least 55 μm in *R. waitii*, based on lengths of incomplete spermatozoa and of the other major components.

Endpiece

Fig. 3 c indicates that the axoneme extends posteriorly beyond the termination of the fibrous sheath where, surrounded simply by the plasma membrane, it forms the endpiece. It is notable that the palisade of microtubules extends to the endpiece.

DISCUSSION

As noted in the Introduction, the phylogenetic affinities of Typhlopidae are poorly understood, with some uncertainty over both their current association with other snakes in the taxon Serpentes [9, 14], and even their current inclusion within Squamata [17]. Recent cladistic analyses of spermatozoal evolution among higher vertebrates [8, 10, 11] provide a useful framework against which to assess the broad scale phylogenetic affinities of the Typhlopidae, based on a previously unexamined aspect of their morphology.

Compared with the various major groups of living amniotes (turtles, crocodiles, birds, tuatara, mammals, squamates) spermatozoa of *Ramphotyphlops* species are, overall, most similar to those of other squamates as described by FURIERI [6] and more recent workers [11, 12, 15].

Some of the features shared by *Ramphotyphlops* spp. (e.g. the presence of an elongate and relatively straight sperm head) are undoubtedly plesiomorphic for amniotes as a whole. However, other features appear to represent derived or apomorphic characters of possible phylogenetic significance. These apomorphic, squamatan characteristics include: the paracrystalline nature of the subacrosomal cone; the absence of an endonuclear canal; the presence of electron dense intermitochondrial "rings" or "plaques"; the extension of the fibrous sheath into the midpiece ([8, 10], see also JAMIESON, Squamates, this volume); and reduction or loss of the annulus. The occurrence of these features in the spermatozoon of *Ramphotyphlops* species provides strong independent confirmation that Typhlopidae are correctly placed within Squamata.

Analysis of phylogenetic relationships within Squamata based on spermatozoal characters is hindered by the many gaps in current taxonomic coverage. Among the snakes for example, comparable observations are available only for representatives of three other families, Colubridae [1, 6, 7, 16, 18, 19], Viperidae [6] and Boidae [2,7], and no data are available for more than a dozen other families. Similarly, little or no information is available for many important groups of lizards, including some which have been putatively implicated in the evolution of snakes (e.g. varanoids [14], dibamids [4]).

Among the various squamate groups for which spermatozoal data are available, *Ramphotyphlops* spermatozoa appear particularly close to those of snakes. In all, five features appear to be uniquely shared among the various families of snakes, namely: (1) the extreme forward prolongation of the fibrous sheath to the level of the neck; (2) the presence of a distinct neck cylinder; (3) the multi-layered nature of the midpiece plasma membrane; (4) the extremely elongate nature of the individual mitochondria; and (5) the extreme elongation of the midpiece. Each of these features appears to represent a derived or apomorphic character relative to a basal squamate condition [8, 10] and they thus collectively support the current placement of Typhlopidae within Serpentes.

Ramphotyphlops spermatozoa differ from those of other snakes in the arrangement of mitochondria in the midpiece. For other species of snakes, the mitochondria are variably described as "very slender and rather convoluted" [6] and as "highly contorted and variable in their orientation ... (but with) .. a predominant direction .. in a loose spiral around the flagellum"

Source : MNHN, Paris

[7]. These observations are confirmed by our own unpublished findings for various species of boids, elapids and a viperid. Clearly they are in striking contrast with the highly regular arrangement described for *Ramphotyphlops*. Nevertheless, JAMIESON [Squamates, this volume] has identified zigzagged mitochondria as a characteristics of snakes, also seen in pygopodids.

The mitochondria of lizards and other groups of reptiles (turtles, crocodiles, tuatara etc.) are even less similar to those of *Ramphotyphlops*. In these groups, typically the mitochondria are small, rounded or brick-shaped bodies, arranged either in regular rows intercalated between complete intermitochondrial rings, or, in irregular fashion with scattered intermitochondrial bodies [6, 11, 12, 15]. Exactly how the mitochondria in *Ramphotyphlops* come to be so elongate and furthermore, how they come to be deployed in such a precise and unusual manner, are clearly issues of general interest which should be further investigated through examination of the spermiogenetic process.

Another difference between spermatozoa of *Ramphotyphlops* and those of other snakes concerns the granular layer external to the principal piece fibrous sheath. This material appears to be absent from the spermatozoa of both boid and colubroid snakes, among which the flagellum shows a more pronounced narrowing at the annulus. Among other squamates, a granular layer is present in the principal piece of at least some skinks [11, 12] and varanids [unpublished data]. Hence this may represent a plesiomorphic feature within Squamata.

Significant differences in spermatozoal structure were observed between each of the three species of *Ramphotyphlops* examined in this study. Of these, the most striking differences concern the presence and electron density of the intermitochondrial bodies of the midpieces: conspicuous, but relatively few, electron dense bodies in the midpiece in *R. endoterus* and granular structures of low electron density in *R. waitii* and *R. australis*. These appear to be even less common in the midpiece than they are in *R. endoterus*. Interestingly, these differences in the relative electron density correlate with that observed in the neck cylinder; dense in *R. endoterus* and of low electron density in the other species. The possibility that these characteristics are related is further strengthened by HAMILTON & FAWCETT's [7] finding that the neck cylinder and the intermitochondrial plaques both arise from a common body of granular material which lies in close topographical relationship to the ends of mitochondria as they assemble around the distal centriole and base of the axoneme during late spermiogenesis. However, because this observation is at odds with recent claims that derive the intermitochondrial bodies directly from mitochondria [3, 8], we defer any further speculation pending analysis of spermiogenesis in *Ramphotyphlops*.

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Source : MNHN, Paris

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Source : MNHN, Paris

Ultrastructural and Light Microscopic Observations of Mature Epididymal Spermatozoa and Sperm Maturation of the Greater Bilby, *Macrotis lagotis* (Metatheria, Mammalia)

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ABSTRACT

Light microscopic and ultrastructural observations were made on mature *Macrotis lagotis* cauda epididymal spermatozoa. Sperm nuclear length measures 13.2 µm, midpiece length 16.2 µm and total sperm length 149.4 µm. The sperm head is simple and without lateral concavities typical of the Peramelidae. Parachromatin-like material is present as a thin layer on the dorsal nuclear surface and the lateral margins of the sperm head are uncondensed and pitted. The acrosome covers 2/5 of the dorso-rostral nuclear surface, extending caudally. The mitochondrial sheath is essentially round but slightly flattened in transverse section and sculptured on its inner surface to accommodate dense outer fibres. These fibres are widely separated in the caudal region of the midpiece and connecting lamellae doubled. The annulus is typically perameloid in structure. Unique cauda epididymal sperm characteristics include: lattice substructural material about the neck region and on the caudo-ventral inner surface of the nucleus; arcs of double thickened membranes underlying the plasma membrane near the caudal midpiece and the neck region of axoneme bifurcate about the proximal centriole. Evidence of sperm maturation during epididymal transit includes: dislocation and cranial migration of the neck insertion from a primary implantation fossa to a secondary insertion at the inner tip of the nucleus; slight acrosomal compaction; slight rotation of the sperm head parallel to the longitudinal axis of the sperm and the shedding of a cytoplasmic droplet. Although no cladistic analysis was attempted, the unique characteristics of *M. lagotis* spermatozoa support the present taxonomic status of the Thylacomyidae as a distinct family. In addition, shared spermatological characteristics between that of the Thylacomyidae, Dasyuridae, Peramelidae and *Tarsipes rostratus* are also evidence of a close phylogenetic relationship. However, the precise phylogeny of the Thylacomyidae requires further investigation.

RÉSUMÉ

Observations en microscopie photonique et électronique sur le spermatozoïde mûr de l'épididyme et sa maturation chez le grand Bilby *Macrotis lagotis* (Metatheria, Mammalia).

Des observations en microscopie photonique et électronique ont été faites sur les spermatozoïdes de la queue de l'épididyme de *Macrotis lagotis* (Bilby ou Grand Lièvre Marsupial). Les longueurs du noyau, de la pièce intermédiaire et du spermatozoïde sont de 13.2, 16.2 et 149.4 µm. La tête du spermatozoïde est simple et sans les cavités latérales typiques des Peramelidae. Un matériel de type parachromatinien est présent sous forme d'une couche fine sur la surface dorsale du noyau et les marges latérales de la tête ne sont pas condensées et sont alvéolées. L'acrosome couvre les deux cinquièmes de

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Source : MNHN, Paris

la surface nucléaire dorso-rostrale et s'étend vers la queue. La gaine mitochondriale est essentiellement ronde mais légèrement aplatie en coupe transversale et sculptée sur sa surface interne pour laisser la place aux fibres denses externes. Ces fibres sont largement séparées dans la région caudale de la pièce intermédiaire et les lamelles de connexion sont doublées. L'annulus a une structure pérameloïde typique. Les caractéristiques originales du spermatozoïde épididymaire sont: un matériel à structure de treillis autour de la région du cou et sur la surface intérieure caudo-ventrale du noyau; des arcs de membrane à double épaisseur soulignant la membrane plasmique près de la partie caudale de la pièce intermédiaire; une bifurcation de la région du cou de l'axonème près du centriole proximal. Les preuves de la maturation du spermatozoïde pendant le transit épididymaire sont: la dislocation et la migration vers la tête de l'insertion du cou à partir d'une fossette primaire jusqu'à une insertion secondaire à l'extrémité interne du noyau; une légère compactage de l'acrosome; une légère rotation de la tête du spermatozoïde parallèlement à l'axe longitudinal du spermatozoïde; la perte d'une goutte cytoplasmique. Bien qu'une analyse cladistique n'ait pas été tentée, les caractéristiques originales du spermatozoïde de *M. lagotis* soutiennent la position actuellement reconnue des Thylacomyidae comme une famille distincte. De plus, les caractéristiques spermatologiques partagées des Thylacomyidae, Dasyuridae, Peramelidae and *T. rostratus* sont la preuve de relations phylogéniques proches. Toutefois, la phylogénie précise des Thylacomyidae demande d'autres études.

While spermiogenesis of *Perameles nasuta* has been thoroughly documented, [19-23], observations of perameloid sperm maturation and mature spermatozoa are limited [14]. HARDING *et al.* [14] noted that most descriptions of epididymal sperm structure in the Peramelidae were incomplete or were included as scattered adjuncts in comparative studies [2, 3, 5, 6, 9-12, 16]. Most studies have examined spermatozoa from *P. nasuta* and *Isoodon macrourus*, with only preliminary observations reported for *P. gunni*, *I. obesulus* and *Echymipera rufescens* spermatozoa.

HARDING *et al.* [14] previously described the ultrastructure and epididymal maturation of *P. nasuta* and *I. macrourus* spermatozoa. These authors noted that mature spermatozoa from both species were similar but also displayed a number of distinct apomorphies. They concluded that ultrastructural changes during sperm maturation were not so obvious when compared to other marsupials, except for the dislocation and marked relocation of the neck from its original implantation fossa to a position close to the rostrum of the nucleus.

Preliminary observations made on *Perorcytes longicauda* and *Echymipera kalubae* sperm have indicated that these species conform to the distinctive perameloid pattern with only minor differences in size and shape [14]. The flagellar structure of *M. lagotis* sperm has also been described by HARDING *et al.* [14] who noted that it conformed to the basic perameloid pattern. However, poor fixation of testicular tissue from one animal, and the occurrence of degenerate spermatozoa from another old male, meant that detailed observations of head structure and to a lesser extent midpiece structure were not possible.

Sperm structure is regarded as a conservative taxonomic character, reflecting evolutionary affinities in the Marsupialia [24]. A detailed evaluation of the ultrastructure and epididymal maturation of *M. lagotis* should, therefore, provide valuable insights into the controversial phylogenetic position of the genus *Macrotis*, which to date has been based on skull and dental characters [8], chromosomal [4] and serological evidence [1].

The present paper describes for the first time the ultrastructure of the mature *Macrotis lagotis* spermatozoon as well as morphological changes during sperm maturation. Light microscopic observations of sperm dimensions are recorded.

MATERIALS AND METHODS

A six year old sexually mature captive male *Macrotis lagotis* located at Western Plains Zoo, Dubbo (32°15′S., 148°37′E.), Australia, was euthanased because of a prolonged chronic staphylococcal infection of the hind limbs. The animal was in poor condition and considered surplus breeding stock.

Under general gaseous anaesthesia (Halothane), the caput and cauda epididymides of one testis were removed and diced into 2mm x 2mm blocks and immersed into cold 3 % glutaraldehyde in 0.1 M phosphate buffer with 6% sucrose for approximately 1 h. Samples were then transferred into 0.1 M sucrose in phosphate buffer and transported back to the laboratory (The University of Queensland, St. Lucia) within 24 hours. Samples for electron microscopy were rinsed in phosphate buffer, followed by post-fixation for 80 min in 1% osmium tetroxide in 0.1 M phosphate buffer with 6%

Source : MNHN, Paris

sucrose. Specimens were then washed in buffer, dehydrated through an ascending ethanol series, infiltrated and embedded in SPURR's low viscosity epoxy resin. Ultrathin sections, 60 to 80 nm, were cut on an LKB 2128 UM IV ultramicrotome. Sections were collected on carbon stabilised colloidion coated 200 mesh and single slot grids and stained with REYNOLD's lead citrate [18], 6% uranyl acetate and further lead citrate [7]. All micrographs were taken on a Hitachi H-300 electron microscope at 75KV.

The epididymidis of the remaining testis was then dissected into warm saline and gently teased to release motile spermatozoa. Nigrosin - eosin stained spermatozoa were used to measure the midpiece and total sperm length via bright-field microscopy at 400 x. As the marsupial sperm nucleus decondenses on air drying [6], measurements of head length and width were made on formalin fixed spermatozoa, using Nomarski differential interference microscopy at 1000 x. All measurements were made using a calibrated eyepiece micrometer.

RESULTS

Light microscope studies: sperm dimensions

Table 1 shows the sperm dimensions recorded for 100 *Macrotis lagotis* spermatozoa.

TABLE 1. — Dimensions of mature epididymal spermatozoa of *Macrotis lagotis*

Sperm dimension	(μm)
Sperm nuclear length	13.2 ± 0.2
Sperm nuclear width	1.6 ± 0.1
Nuclear length: width ratio	8.25
Midpiece length	16.2 ± 0.1
Principal piece length	130.2 ± 0.1
Total sperm length	149.4 ± 0.1

Ultrastructure of mature epididymal spermatozoa

The sperm head of *Macrotis lagotis* is cuneiform-shaped but slightly dorsoventrally flattened (Fig. 1b-c). A deep longitudinal groove runs caudally along the ventral nuclear surface from where the neck is inserted. The nuclear groove is narrow cranially but widens caudally to accommodate the midpiece (Fig. 1a). The majority of the dorsal aspect of the sperm head overlies the anterior 3/5 of the midpiece. Serial transverse sections through the sperm head indicate the presence of a dorsal nuclear ridge which extends from the tip of nucleus to its caudal extremity, but it is not prominent cranially (Fig. 1d-h).

The nucleoplasm is primarily condensed. However, some transverse sections indicate nuclear indentations along the periphery of the ventral flanges of the nucleus (Fig. 1f). A thin layer of parachromatin-like material extends caudally from the rostrum for approximately two fifths the length of the nucleus, and consists of material which is distinct from that of the acrosome, but is less electron dense than the nucleus (Fig. 2f-g). Poor preservation of membrane ultrastructure makes it difficult to ascertain whether the parachromatin-like material is within or outside the nuclear membrane.

There is an original primary and a distinct secondary implantation fossa (Fig. 2f-g). The original implantation fossa is located deep within the sperm head and lined by a thick layer of material which appears to form a basal plate. In the vicinity of the second implantation fossa and surrounding the neck region, granular material which has an ordered substructure is present (Fig. 1f). Some micrographs also indicate the presence of electron dense material which is confined to the caudo-ventral surface of the sperm head in association with the midpiece, (Fig. 1a, f), however, this material is particularly prominent in immature spermatozoa (Fig. 3b). It appears to have a distinct "honeycomb" lattice substructure.

Source : MNHN, Paris

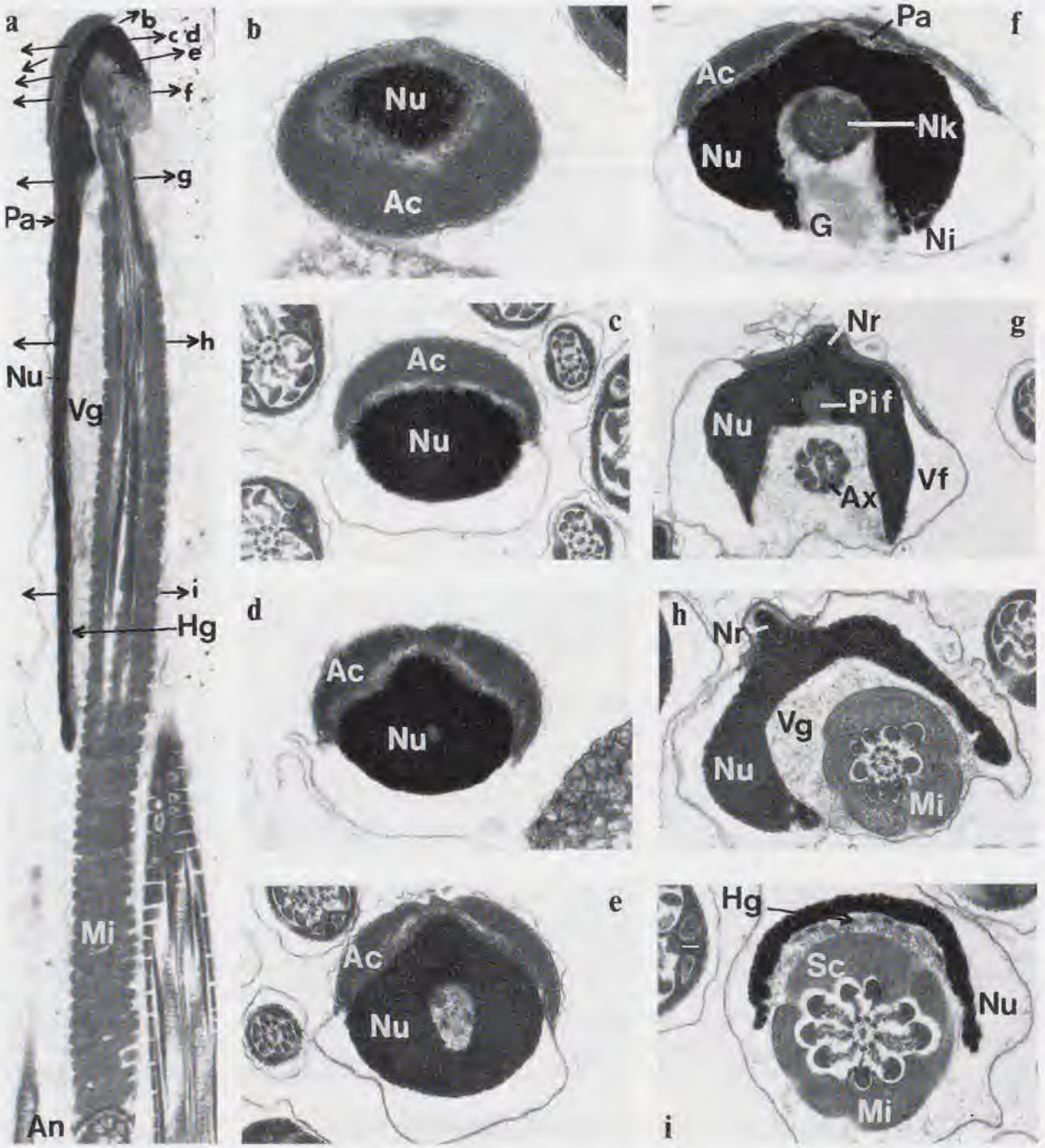


FIG. 1. — Electron micrographs of longitudinal sections (LS) and transverse sections (TS) of the nuclear and midpiece region of *Macrotis lagotis* cauda epididymal spermatozoa. **a:** LS of head and midpiece (x 11 200). **b-i:** TS through head to midpiece regions of *M. lagotis* spermatozoa as indicated in Fig. 1a. **a-e:** nuclear acrosomal region; b, x 21 000; c, x 21 000; d, x 21 000; e x 28 000; **f, g:** neck region; f, x 22 000; g, x 16 500. **h, i:** midpiece region; h, x 22 000; i, x 22 000.

Source : MNHN, Paris

The majority of the acrosomal matrix of the mature acrosome forms a rudimentary cap over the cranial dorsal nuclear surface (Figs 1a, 2f-g). However, it also extends dorso-caudally for two fifths the length of the nucleus in close association with the parachromatin-like material. Serial transverse sections through the caudal region of the acrosome indicate that the distribution of the acrosome conforms to the underlying profile of the nucleus (Fig. 1b-f). The cranial margin of the acrosome forms a lip at the cranial extremity of the nucleus (Figs 1a, 2f-g, 3a-b).

Longitudinal sections reveal that the axoneme terminates in a bifurcated structure, with the proximal centriole situated within the fork of the bifurcation (Fig. 2f). The dorsal arm of the bifurcation extends cranially past the proximal centriole and terminates surrounded by electron-lucent material. The ventral arm of the bifurcation is also surrounded by electron-lucent material but extends only adjacent to the proximal centriole.

The midpiece and underlying mitochondrial sheath are slightly flattened in transverse section (Fig. 2b). The ratio of the cross sectional diameters perpendicular and parallel to the centrally located axonemal fibres is 1.08 ± 0.03 (n=16). Mitochondria in transverse section are sculptured on their inner surfaces to accommodate the displaced outer fibres (Fig. 2b). Dense outer fibres 3 and 8 have an evenly circular contour, while the rest of the fibres has a semi-circular contour in cross section (Fig. 2b). The dense outer fibres of the cranial extremity of the axoneme, in the vicinity of the midpiece, are positioned close to the underlying axoneme doublet. However, in all transverse sections of the axoneme caudal to the nucleus, the dense outer fibres become widely separated. The separation is greatest between dense outer fibres 1, 5 and 6 and least between 3 and 8. Connecting lamellae in *M. lagotis* are double in nature except for fibres 3 and 8 (Fig. 2b). There is no diffuse granular material surrounding the outside of the mitochondrial sheath. However, transverse sections of the caudal midpiece region reveal an arc of doubled thicked membrane structures underlying the plasma membrane in the region of dense outer fibres 2, 3 and 4 and 7, 8 and 9 (Fig. 2b). In longitudinal sections these structures are located from the terminal caudal portion of the midpiece to a position approximately 10 mitochondrial whorls cranially. The annulus forms a fibrous ring below the most caudal mitochondria, and is joined to the fibrous sheath of the principal piece by an array of fine fibres (Fig. 2a).

Serial sections of the cranial region of the principal piece of *M. lagotis* spermatozoa are slightly flattened in cross section (Fig. 2c). The ratio of the cross sectional diameters perpendicular and parallel to centrally located axonemal fibres is 1.04 ± 0.02 (n=47). The fibrous sheath had only one fenestration (vacuole) in each rib on either side of the longitudinal columns (Fig. 2c). There is no typical endpiece, as the axoneme terminates before that of the fibrous sheath (Fig. 2d). Surrounding the fibrous sheath and underlying the plasmalemma there is also a layer of fine material; the accessory sheath (Fig. 2e).

Epididymal sperm maturation

Light microscopic observations of spermatozoa in the lumen of the seminiferous tubule at spermiation and the caput epididymides indicate that the long axis of the nucleus ranges from perpendicular (Fig. 3a) to almost completely rotated parallel to the long axis of the axoneme (Fig. 3b). However, by the time spermatozoon reaches the cauda epididymidis the nucleus has fully rotated and is streamlined with the tail. The most notable change during epididymal transit is the dislocation and migration of the neck region from the original primary implantation fossa to a secondary implantation fossa located cranially (Fig. 3b). The dorso-cranial portion of the acrosome of caput epididymal spermatozoa appears button-like (Fig. 3c). However, during epididymal maturation this region appears to undergo compaction; becoming slightly flattened. The cytoplasmic droplet of the caput epididymal spermatozoon is eccentrically placed to one side

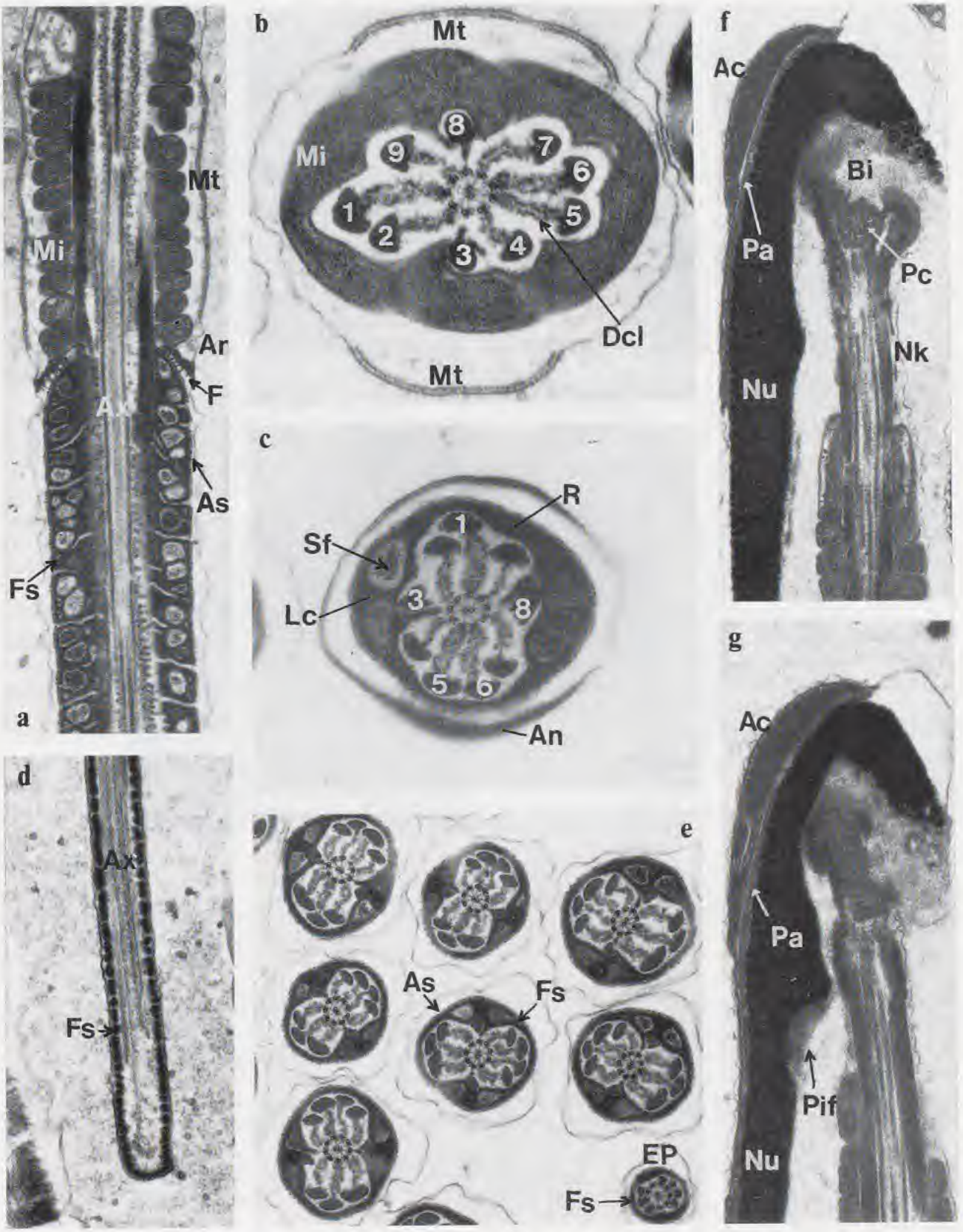
Source : MNHN, Paris

TABLE 2. — Ultrastructural characteristics of *M. lagotis* cauda epididymal spermatozoa and sperm maturation as compared with the Peramelidae and Dasyuridae. Bold type = *M. lagotis* unique character. Italics = *M. lagotis* shared character

Sperm Characteristics	Peramelidae	*Macrotis lagotis*	Dasyuridae
1. Sperm head shape	Complex	*Simple*	*Simple*
2. Presence of parachromatin	Extensive	?	?
3. Extent of ventral groove and relative position of neck insertion of mature spermatozoa	*Elongated and high*	*Elongated and high*	Moderate
4. Modification of nuclear periphery	*Only at lateral margins*	*Only at lateral margins*	Marked indentations on ventral surface
5. Lattice substructural material in region between the nucleus and the neck	No	**Yes**	No
6. Dorsal nuclear ridge	Yes but flattened	Yes but pointed	No
7. Head length (µm)	5.7 - 6.0	13.2 ± 0.2	9.5 - 12.7
8. Acrosome coverage as a fraction of the nucleus	Rostrum only	**2/5 of nuclear surface**	4/5 of nuclear surface
9. Shape of mitochondrial sheath in transverse section	Circular	**Slightly flattened**	Markedly flattened
10. Granular material surrounding mitochondrial sheath	Yes	*No*	*No*
11. Helical fibre network in midpiece region	*Absent*	*Absent*	Present
12. "Honey-comb" arranged lattice material on the caudo-ventral inner surface of the nucleus	Absent	**Present**	Absent
13. Arc of double thickened membrane underlining the plasma membrane of the caudal midpiece in the region of dense outer fibres 2,3,4 and 7,8,9.	No	**Yes**	No
14. Midpiece length (µm)	10.7 - 19.3	16.2 ± 0.2	34.0 - 40.0
15. Neck region of axoneme bifurcated about the proximal centriole.	No	**Yes**	No
16. Shape of dense outer fibres in transverse section.	*Semi-circular*	*Semi-circular*	Circular
17. Connecting lamellae: (double or single)	*Double*	*Double*	Single
18. Shape of transverse section of principal piece	Diamond	**Slightly flattened**	Markedly flattened
19. Double or single fenestration in longitudinal columns	Double	*Single*	*Single*
20. Accessory sheath of principal piece	*Present*	*Present*	Absent
21. End Piece - lack of axoneme in terminal portion	*Yes*	*Yes*	?
22. Total sperm length (µm)	171.1 - 199.8	149.4 ± 0.1	218 - 255
Sperm maturation			
23. Neck dislocation from primary implantation fossa and cranial migration	*Yes, extreme*	*Yes, extreme*	No
24. Extent of head rotation	*Slight*	*Slight*	Marked
25. Acrosomal compaction	Not obvious	**Slight**	No
26. Increase in the electron density of the mitochondrial matrix material	Yes	*No*	*No*
27. Development of helical network surrounding the mitochondria and underlining the midpiece plasma membrane	*No*	*No*	Yes

FIG. 2. — Electron micrographs of *Macrotis lagotis* cauda epididymal spermatozoa. **a:** LS of midpiece and principal piece region illustrating microtubular structures at the caudal extremity of the midpiece and the annulus (x 22 500). **b:** TS through caudal extremity of midpiece (x 39 000). **c:** TS through annular region (x 36 000). **d:** LS of endpiece (x 28 000). **e:** TS through principal and endpiece regions (x 14 000). **f:** LS of the nuclear-flagellar connection, illustrating bifurcated neck-piece (x 24 000). **g:** LS of the nuclear-flagellar connection, illustrating primary implantation fossa (x 24 000).

Source : MNHN, Paris



Source : MNHN, Paris

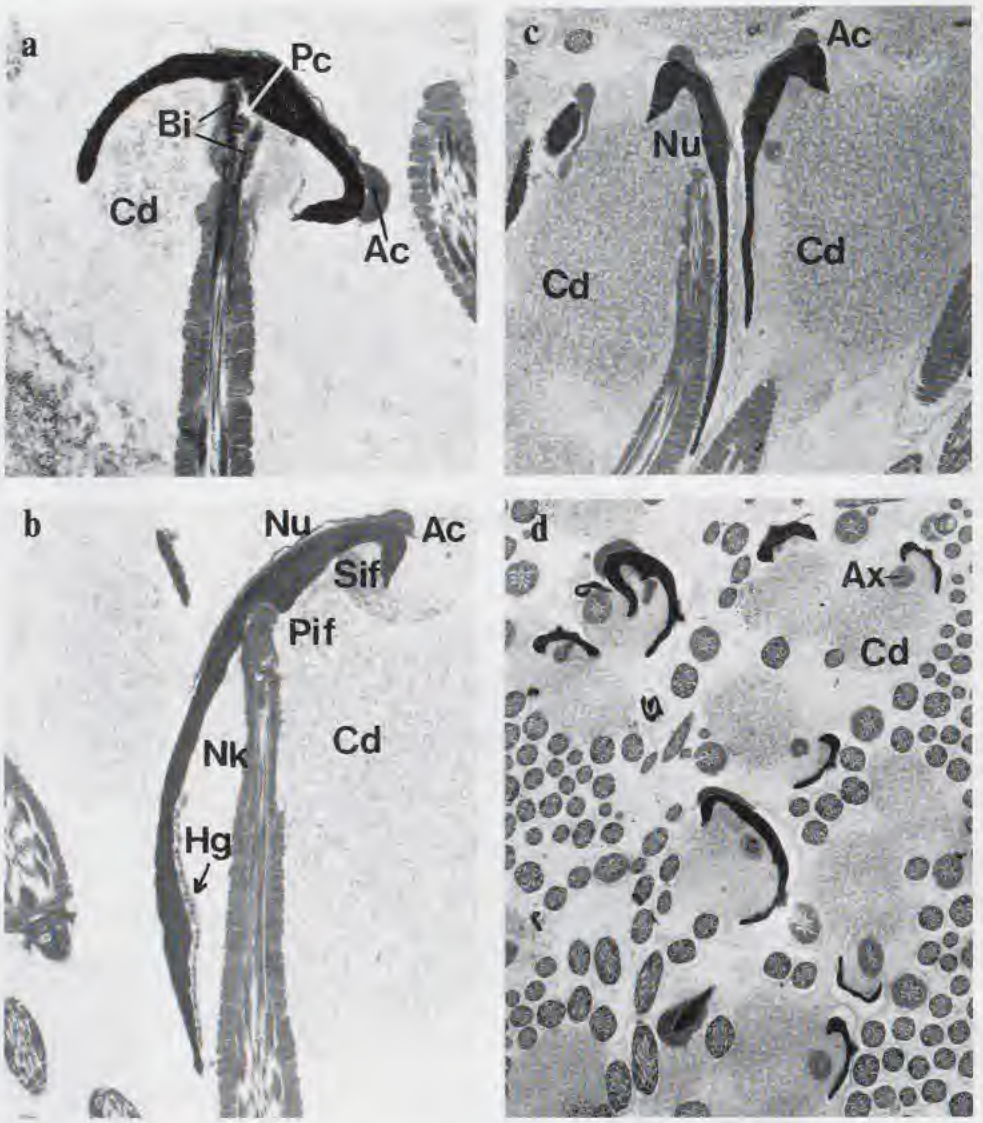


FIG. 3. — Electron micrographs of *Macrotis lagotis* immature caput epididymal spermatozoa. **a, b:** LS illustrating neck insertion into primary implantation fossa, button-like acrosome and cytoplasmic droplet. Also note bifurcated neck in a and honey-comb granular material in the caudal extremity of the nucleus in b. **c:** LS of nuclear-flagellar connection highlighting eccentric position of the cytoplasmic droplet. **d:** TS low power electron micrograph of immature spermatozoa. a, x 9 800; b, x 9 100; c, x 5 500; d, x 2 800.

Abbreviations for all figures: Ac, acrosome; An, annulus; As, accessory sheath; Ax, axoneme; Bi, bifurcated neck; Cd, cytoplasmic droplet; Dcl, double connecting lamellae; EP, endpiece; F, fibres of annulus attached to fibrous sheath; Fs, fibrous sheath; G, granular material about neck region; Hg, honey comb granular material; Lc, longitudinal columns; Mi, mitochondria; Mt, double thickened membrane; Ni, nuclear indentations; Nr, nuclear ridge; Nu, nucleus; Pa, parachromatin-like material; Pc, proximal centriole; Pif, primary implantation fossa; R, ribs of fibrous sheath; Sc, sculptured mitochondria; Sif, secondary implantation fossa; Sf, single fenestration of the fibrous sheath; Vf, ventral flange of nucleus; Vg, ventral groove ; 1-9, relative position of dense outer fibres.

of the midpiece and sperm head (Fig. 3a-d). The droplet appears to consist of two types of electron dense material. The first is easily recognised as composed of cytoplasmic membrane remnants, while the second is of less dense consistency (Fig. 3c).

Source : MNHN, Paris

DISCUSSION

Macrotis lagotis is the only extant member of the Thylacomyidae. Its spermatozoa are large compared to those of other mammals, but smaller than those of the Dasyuridae, Peramelidae and *Tarsipes rostratus*. Interestingly, while the total length of the *M. lagotis* spermatozoon is shorter than that of the other perameloids, its head length is up to twice as long.

Spermatozoa of *M. lagotis* display numerous unique ultrastructural characteristics which differentiate them both from the Peramelidae, (traditionally regarded as sharing a close phylogenetic relative), and the Dasyuridae. There are also numerous shared sperm characteristics between *M. lagotis* and the Dasyuridae and Peramelidae (See Table 2).

The head shape of *M. lagotis* is simple in profile, characteristically marsupialian [14] and without the complicated lateral concavities of typical Peramelidae or the hooked profile of the Vombatidae and Phascolarctidae. Like the Peramelidae, the ventral groove of sperm of *M. lagotis* is extensive, reaching almost to the tip of the nucleus. Similarly, both *Isoodon macrourus* and *M. lagotis*, have a nuclear ridge or keel on the dorsal surface of the sperm head, although the ridge of *I. macrourus* appears somewhat more flattened [14]. It is possible that such a structure could provide hydrodynamic stability during sperm motility.

The sperm nucleus of the Peramelidae is characterised by two chromatin components; an electron-dense and an electron-lucent component (or parachromatin). The nuclei of sperm of *M. lagotis* also appeared to contain parachromatin-like material; however, it is confined to the dorsal surface of the sperm head. It is still equivocal whether this material is part of the nucleoplasm. The occurrence of indentations along the periphery of the ventral flanges of the sperm nucleus appears similar to that described in the Peramelidae [14] but they are not as marked as those found in the Dasyuridae [15]. The anterior extremity of the ventral groove is also surrounded by material which is less electron dense than that of the parachromatin-like material. Unique to *M. lagotis*, so far as is known, this material had a lattice substructure.

During sperm maturation the neck of the axoneme dislocates from the primary implantation fossa to become located deep within the cranial extremity of the ventral groove. The secondary implantation fossa, however, does not possess a basal plate, and like other perameloid marsupials, connecting structures are not evident in association with the neck [14]. HARDING *et al.* [14] commented that lack of connecting structures might have meant that the head-flagella connection of the mature spermatozoon is not secure and that the stream-lined alignment of head and flagellum probably aided in preventing separation. Interestingly, this study also reveals electron dense material with a honey-comb lattice substructure on the interior surface of the caudo-dorsal sperm nucleus. This material is also apparent in nigrosin-eosin stained spermatozoa. Given that the sperm head of *M. lagotis* is found to be twice as long as that of other perameloid marsupials, it is possible that this material cements the caudo-dorsal extremity of the nucleus to the midpiece region, thus adding further strength to the head-flagella connection. One of the most striking features of the *M. lagotis* spermatozoon is the bifurcated morphology of the connecting piece, with the proximal centriole located within the fork of the bifurcation. This unusual structure appears to be unique to *M. lagotis* and a functional significance is elusive. Perhaps such an arrangement also strengthens the head-flagellum connection.

Acrosomal morphology of *M. lagotis* appears most similar to that of *T. rostratus* (Tarsipedidae). HARDING *et al.* [13] noted that the acrosome of *T. rostratus* covered the dorsal surface of approximately the anterior 2/3 of the nucleus. This observation compares with 2/5 of the nucleus of *M. lagotis* reported in this study. Secondly, the acrosome of both *T. rostratus* and *M. lagotis* forms a small lip at the cranial tip of the nucleus (HARDING, pers. comm.). The caudal portion of the acrosome of spermatozoa in the caput epididymis appears button like, but during epididymal transit the acrosome in this region flattens. Although acrosomal maturation of *M. lagotis* is only comparatively minor, perameloid and dasyuroid acrosomes appear fully compacted prior to epididymal transit.

Source : MNHN, Paris

HARDING *et al.* [14] had previously described the sperm flagellar ultrastructure of *M. lagotis*, noting the wide separation of dense outer fibres from the axoneme, presence of paired connecting lamellae, the single fenestration on either side of the longitudinal columns and the lack of a diamond shaped transverse flagellar section with a concave profile. However, owing to poor fixation and consequent quality of the material examined, midpiece ultrastructure was not described.

As for other perameloid marsupials [14], the dense outer fibres of *M. lagotis* in the cranial portion of the midpiece lie close to the underlying axoneme (Fig. 1h), but in caudal regions of the midpiece the dense outer fibres become radially displaced. However, the extent of the displacement in *M. lagotis* and the Peramelidae is not as marked as in the Dasyuridae and *T. rostratus* [13, 15]. As in the other perameloid marsupials [14] the shape of the dense outer fibres of *M. lagotis* sperm (except 3 and 8) is semi-circular in transverse section.

The shape of the caudal midpiece of *M. lagotis* in transverse section is slightly flattened, unlike that of the other perameloid marsupials which are essentially circular [14]. Also in common with the perameloids, dasyuroids and *Tarsipes rostratus*, the mitochondria are sculptured on their inner surface to accommodate the displaced dense outer fibres [13, 14, 15]. Spermatozoa of *M. lagotis* lack the granular layer surrounding the mitochondrial sheath, which is typical of the perameloid midpiece. However, there is an arc of double thickened membrane material underlying the plasma membrane in the region of dense outer fibres 2, 3 and 4 and 7, 8 and 9. These structures appear to be unique to *M. lagotis* . Observations from the midpiece region of *M. lagotis* sperm failed to detect the presence of an electron dense plate surrounding the midpiece or an underlying granular wedge; both apomorphies which characterize other perameloid spermatozoa [14].

The annulus of *M. lagotis* spermatozoa is "perameloid-like" in forming a fibrous ring caudal to the most posterior mitochondria and is joined to the fibrous sheath of the principal piece by an array of fine fibres [14]. Also similar to the sperm of other perameloids, dasyuroids and *T. rostratus*, the plasma membrane in the region of the annulus is not indented.

Compared to the other perameloids, dasyuroids and *T. rostratus* [13-15], transverse sections of the cranial principal piece are markedly less flattened: a feature unique to *M. lagotis*. However, unlike the other perameloids, but similar to the dasyuroids, the fibrous sheath of *M. lagotis* has only one fenestration in the rib of either of the longitudinal columns. In common with the Peramelidae, *M. lagotis* also has an accessory sheath surrounding the fibrous sheath of the principal piece, and the axoneme terminates before the fibrous sheath [14].

In conclusion, *Macrotis lagotis* spermatozoa display a number of unique characters that appear to separate them from the Peramelidae, Dasyuridae and *Tarsipes rostratus*. While a detailed cladistic analysis is currently in preparation, ultrastructural spermatozoal evidence on *M. lagotis* reported in this study, appears to support the present taxonomic status of the Family Thylacomyidae and confirm the close phylogenetic affinities (intermediate) with the Peramelidae, Dasyuridae and *T. rostratus*.

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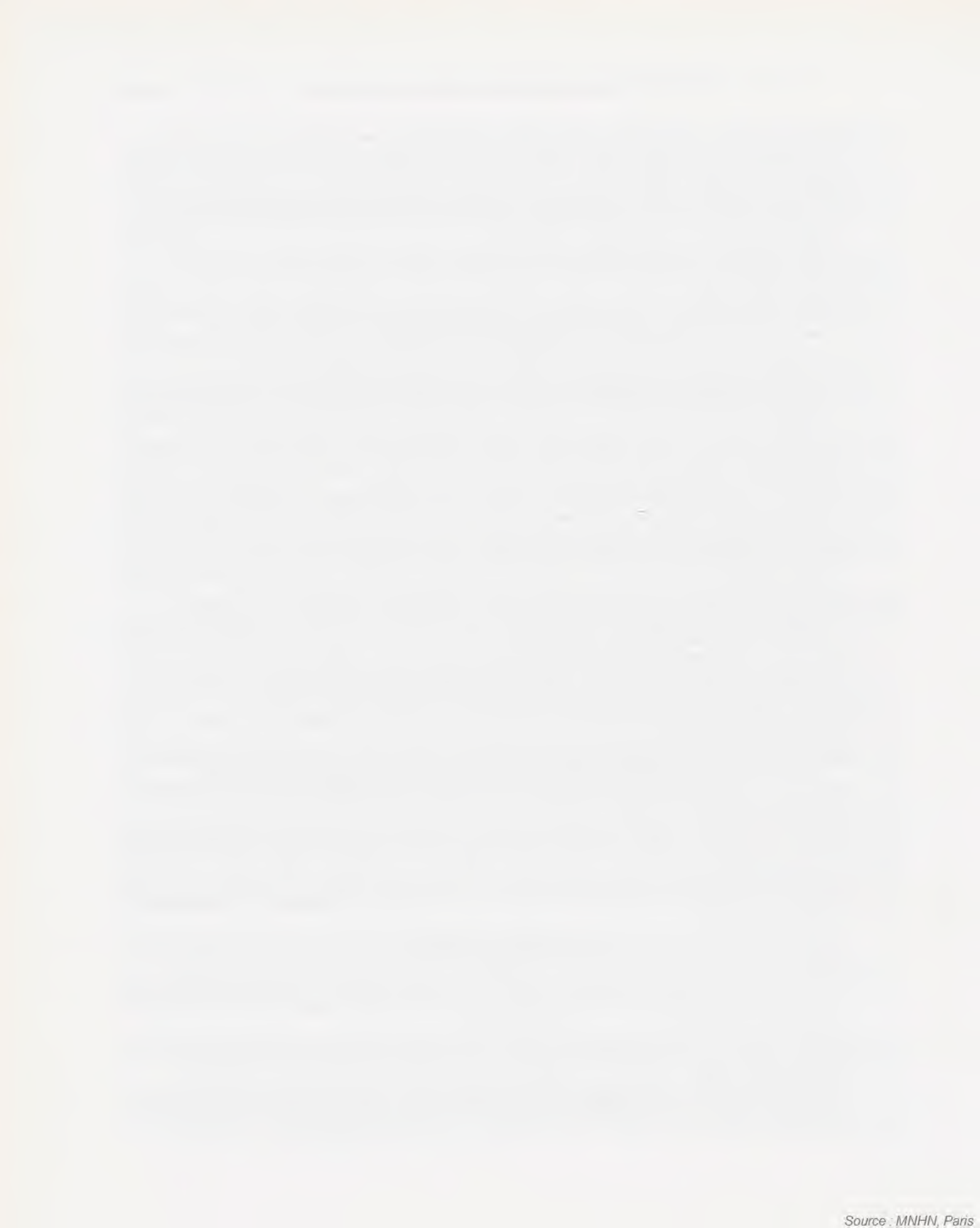
Source : MNHN, Paris



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Variation in Sperm Head Morphology of Muroid Rodents of Africa: Phylogenetic Implications

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ABSTRACT

Sperm morphology from individuals of the following subfamilies of muroid rodents of southern Africa is determined: Cricetomyinae (*Saccostomus*), Gerbillinae (*Gerbillurus* and *Tatera*), Dendromurinae (*Dendromus*, *Malacothrix*, *Prionomys*, *Steatomys*, and *Deomys*), and Otomyinae (*Otomys*); *Mystromys* (subfamily Mystromyinae) is used as an outgroup. The sperm head of most species including that of *Mystromys* is falciform in shape but differences in internal organisation occur. In *Mystromys* it is long and thin and there is a very large apical acrosomal segment. The falciform sperm heads of *Saccostomus* and *Malacothrix* are broader basally but have a similar organisation apically, whereas in *Deomys* the sperm head is very small, bilaterally flattened, with no apical hook. *Gerbillurus* sperm head terminates in a sharp pointed apex, whereas in *Tatera* it is round apically and a deep invagination occurs in the caudal nuclear region. In *Otomys* the sperm head is falciform and the organisation of the perforatorium and acrosome is similar to that of most murine rodents apart from *Acomys* and *Uranomys*. This study suggests that a falciform sperm type is probably the ancestral condition for the dendromurine-cricetomyine-otomyine-murine clade. Sperm of both *Deomys* and *Tatera* are highly divergent, and those of the otomyines and murines are very different from sperm of species in other subfamilies; since a very similar or identical morphology occurs in most species of the Otomyinae and Murinae it suggests that these two subfamilies are sister groups to the exclusion of the other subfamilies and of *Acomys* and *Uranomys*.

RÉSUMÉ

Les variations morphologiques de la tête des spermatozoïdes des Rongeurs Muroïdes d'Afrique: implications phylogénétiques

La morphologie du spermatozoïde a été déterminée chez des spécimens appartenant aux familles suivantes de Rongeurs Muroïdes d'Afrique du Sud : Cricetomyinae (*Saccostomus*), Gerbillinae (*Gerbillurus* et *Tatera*), Dendromurinae (*Dendromus*, *Malacothrix*, *Prionomys*, *Steatomys* et *Deomys*), et Otomyinae (*Otomys*). *Mystromys* (sous-famille Mystromyinae) a été utilisé comme outgroup. La tête du spermatozoïde de la plupart des espèces, y compris celle de *Mystromys*, est falciforme, mais il existe des différences dans l'organisation interne. Chez *Mystromys* la tête est longue et fine et un grand segment acrosomien apical est présent. Les têtes falciformes de *Saccostomus* et *Malacothrix* sont plus larges à la base mais ont une même organisation à l'apex, alors que la tête de *Deomys* est très petite, aplatie bilatéralement, et sans crochet. La tête de *Gerbillurus* se termine en un apex très effilé, alors que chez *Tatera* la tête est ronde à l'apex et une invagination profonde existe dans la région nucléaire caudale. Chez *Otomys* la tête est falciforme et l'organisation du perforatorium et de l'acrosome est similaire à celle de la plupart des Rongeurs Muridae, à part *Acomys* et *Uranomys*. Cette étude suggère que le spermatozoïde de type falciforme est la condition ancestrale pour le clade Dendromurinae-Cricetomyinae-Otomyinae-Murinae. Les spermatozoïdes de *Deomys* et de *Tatera* sont très divergents, et ceux des Otomyinae et Murinae sont très différents de ceux des espèces des autres sous-familles. Comme cette morphologie très similaire ou identique se trouve chez la plupart des espèces des Otomyinae et Murinae, cela suggère que ces deux sous-familles sont des groupes-frères, à l'exclusion des autres sous-familles et d'*Acomys* et *Uranomys*.

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Source : MNHN, Paris

The superfamily of muroid rodents is composed of over 1300 species of about 280 genera with major radiations in both the New and Old Worlds. SIMPSON [37] proposed two major families: the Cricetidae and the Muridae with the latter including the African climbing mice (Dendromurinae), swamp rats (Otomyinae), as well as true rats and mice of the Old World (Murinae) inclusive of the African pouched mouse *Saccostomus*. Subsequently it was suggested that the Dendromurinae [26] and Otomyinae [28] were closer to the Cricetidae, and that *Saccostomus* and other cricetomyines were also part of this family [31, 32]. CHALINE *et al.* [14] later erected several new families: the Dendromuridae, Cricetomyidae, Nesomyidae (which included the otomyines), whereas REIG [34] proposed these groups as subfamilies within the Cricetidae. CARLETON & MUSSER [12] expanded the family Muridae to embrace 15 subfamilies including the Dendromurinae, Otomyinae, Murinae, Gerbillinae, Cricetinae and others. Over the last 10 years palaeontological and biochemical data have suggested that the dendromurines, cricetomyines, and gerbillines are probably more closely related to each other than any is to the Cricetinae [11, 13, 18].

Apart from the unstable higher order taxonomy, it has also become apparent that at least one genus placed within the Murinae, *Acomys*, may not be part of this group [36, 40]. Recent DNA-DNA hybridisation studies have suggested that it, together with *Uranomys* and *Lophuromys*, form a monophyletic clade that clusters with the Gerbillinae [15, 30], whereas dental features of *Acomys* and *Uranomys* appear to be intermediate between this group and the Murinae [16].

Since the taxonomy and phylogenetic relationships between these major groups of African muroids is controversial, it would seem timely to explore the use of other data sets for hypothesising relationships. Over the last few years spermatozoal morphology has been used with varying degrees of success as an independent character for suggesting relationships between various marsupials [22, 24, 38], bats [23], American cricetids [27], and Asian and Australasian murids [5, 7, 8, 10]. Here it is used to explore relationships between the subfamilies of African murids and, in particular, between members of the Murinae, Dendromyinae, Gerbillinae, Cricetomyinae and Otomyinae subfamilies. As an outgroup comparison, the sperm morphology of *Mystromys* (an archaic African species usually placed in the Mystromyinae) is considered. To date there have been only a few investigations on morphology of spermatozoa of African murid rodents [e.g. 1, 3, 6, 21, 39], and there appear to be no studies using it to determine the relationships between the murid subfamilies. This is the aim of the present study.

MATERIAL AND METHODS

Sperm morphology was investigated by light (LM) and, where possible, transmission electron microscopy (TEM) from: *Mystromys alba* (subfamily Mystromyinae), *Prionomys batesi*, *Steatomys parvus*, *Dendromus mystacalis*, *D. mesomelas*, *Deomys ferrugineus*, and *Malacothrix typica* (subfamily Dendromurinae), *Saccostomus campestris* (subfamily Cricetomyinae), *Otomys irroratus* (subfamily Otomyinae), and *Gerbillurus paeba* and *Tatera leucogaster* (subfamily Gerbillinae). The sources of animals were: *M. alba* and *S. campestris* from the South African Institute for Medical Research, Sandringham, Johannesburg, one *O. irroratus* from near Pietermaritzburg and another from Ngare Forest, Natal Province. *G. paeba* came from near Swakopmond, and a *T. leucogaster* from Etosha pan, Namibia. A second *T. leucogaster* was collected in northern Quazulu. The *M. typica* specimen came from near Hutchinson, Cape Province (Transvaal Museum No 36676), and material from the other dendromurines was from specimens at the Natural History Museum, London which included *D. ferrugineus* from Kivu Province, Zaire, *D. mystacalis* from Babaka, Isai River, Huri, Zaire, *D. mesomelas* from Uganda, and *S. parvus* from Swaziland.

Caudae epididymides were immersed in either 10% phosphate buffered formaldehyde or 3% paraformaldehyde/3% glutaraldehyde made up in 0.1 M phosphate buffer, pH 7.4. For LM, spermatozoa were extruded from the ducts and placed on microscope slides. For TEM the tissue was rinsed in phosphate buffer, fixed in osmium tetroxide, rinsed again, dehydrated by passing through a graded ethanol series, and embedded in Araldite. Thick and thin plastic sections were cut, the latter stained with lead citrate and uranyl nitrate, and observed with a JEOL 100S and/or Philips CM 100 EM.

Source : MNHN, Paris

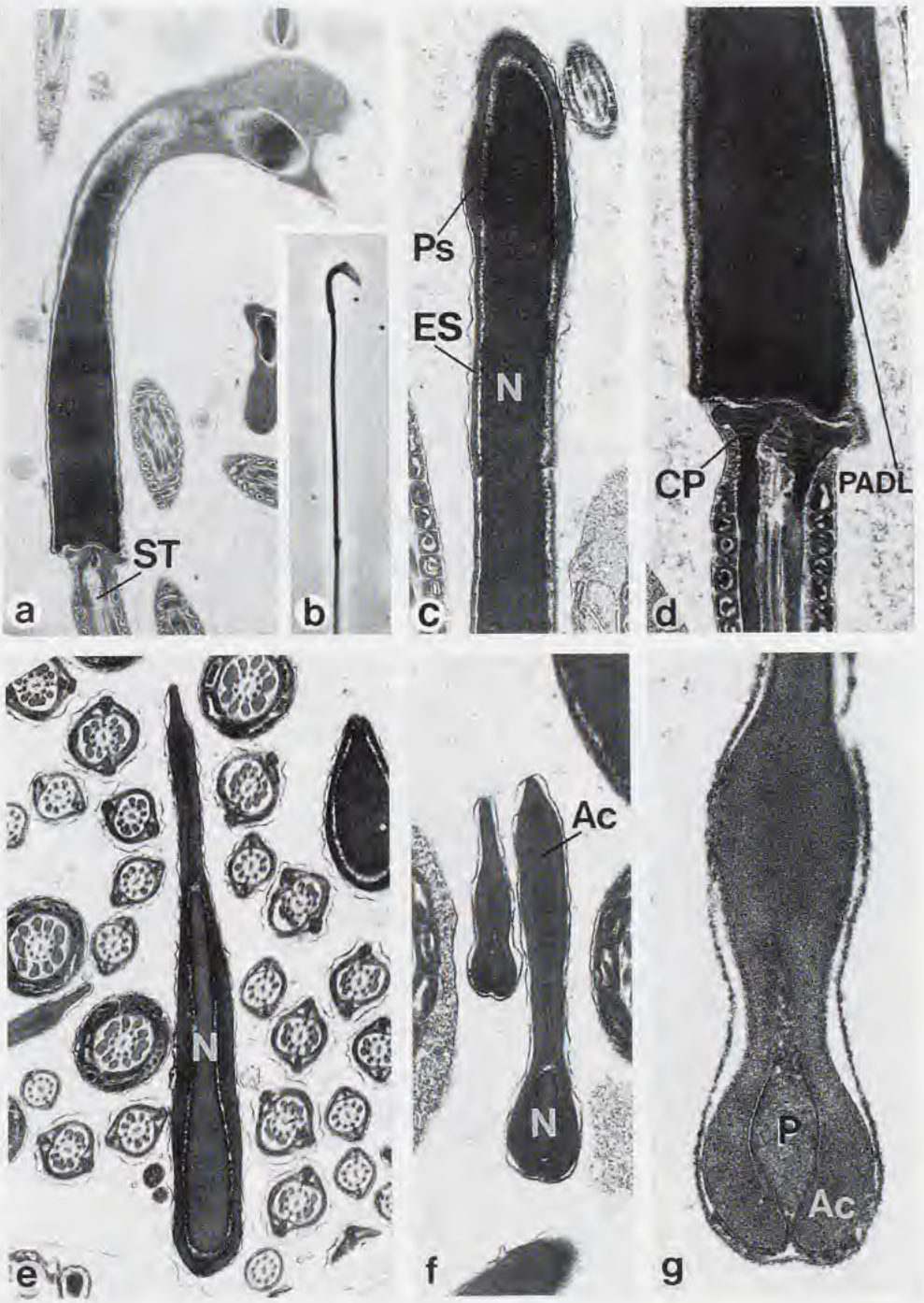


FIG. 1. — **a-g:** Sperm of *Mystromys alba*. **a, b:** Head is falciform. **c:** An electron-dense nucleus (N) is capped by a large acrosome which has a narrow equatorial (ES) and wider principal segment (PS); **d:** a postacrosomal dense lamina (PADL) occurs in the posterior region of sperm head; **e, f:** anterior region of nucleus is bilaterally flattened and capped by a massive apical acrosome segment; **g:** TS of rostral tip indicates perforatorium (P) surrounded by acrosome (Ac) except midventrally. a, x 8 200; b, x 900; c, x 19 300; d, x 20 100; e, x 17 100; f, x 26 800; g, x 97 000.

Source : MNHN, Paris

RESULTS

Terminology used for the planes of section follows that of LALLI & CLERMONT [25]; thus in a falciform sperm head the convex surface is referred to as dorsal and concave as ventral. The space between the inner acrosomal membrane and outer nuclear envelope is the subacrosomal space and, when extensive, the perforatorium. The taxonomy proposed by CARLETON & MUSSER [12] is, in general, used although the family name of Cricetidae [37] is also referred to.

Mystromyinae

Mystromys alba.The sperm head is falciform with a length of 8-9 µm, maximum breadth of only 1.5 µm, hook length of 5 µm, and connecting piece of tail attaches midbasally with midpiece length of 38 µm and principal and end piece of about 100 µm (Fig. 1a, b).

The bilaterally flattened head has a homogeneous, electron-dense, nucleus that passes into the apical hook (Fig. 1a) which is capped by an acrosome that has a thicker principal than equatorial segment caudal to which is a postacrosomal dense lamina (Fig. 1c, d).

In the anterior region of the sperm head there is a massive apical acrosomal segment that extends at least 1 µm beyond the convex nuclear margin. The acrosomal matrix is more electron-dense medially (Fig. 1e, f), and anterior to the convex nuclear surface, beneath the inner acrosomal membrane, a subacrosomal space with electron-dense material is present (Fig. 1f).

The acrosome and perforatorium extend beyond the rostral tip of the nucleus. Cross sections demonstrate that the acrosome is bilaterally flattened and surrounds the perforatorium, except mid ventrally (Fig. 1g); the perforatorium is spear-shaped in cross section and the anterior region of acrosomal segment widens slightly before tapering apically (Fig. 1g).

Gerbillinae

Gerbillurus paeba. Sagittal (Fig. 2a, b) and frontal (Fig. 2c) TEM sections of the sperm head show that it is broad basally and narrows apically. The connecting piece of the sperm tail attaches to an off-centre basal position. There is a large apical acrosomal segment with a narrower principal segment over the anterior part of the nucleus posterior to which the equatorial segment passes down over much of the convex margin of the nucleus with the postacrosomal dense lamina passing around the posterior caudal margin (Fig. 2a). On the concave surface the equatorial segment is restricted anteriorly (Fig. 2b). Frontal sections through the anterior region of the sperm head indicate an invagination of the inner acrosomal membrane within which electron-dense material of the subacrosomal space occurs (Fig. 2c).

Tatera leucogaster. The sperm morphology of this species is highly divergent. Longitudinal TEM sections indicate that sperm head is bilaterally flattened (Fig. 2d), and has a deep invagination posteriorly (Fig. 2d, e, f). The nucleus tapers apically and its anterior four-fifths is capped by a nearly symmetrical acrosome (Fig. 2d) most of which is composed of a principal segment with the equatorial segment forming a girdle around the posterior nuclear region. The postacrosomal dense lamina is very short. A modest subacrosomal space is present with a small apical extension (Fig. 2d). A complex structural organisation of the connecting piece occurs with the basal plate running parallel to the long axis of the sperm head, and laterally an extension of the nuclear envelope within which material that exhibits light and dark banding occurs (Fig. 2f). The capitulum of the connecting piece passes vertically up into the implantation fossa and narrows apically (Fig. 2f).

Source : MNHN, Paris

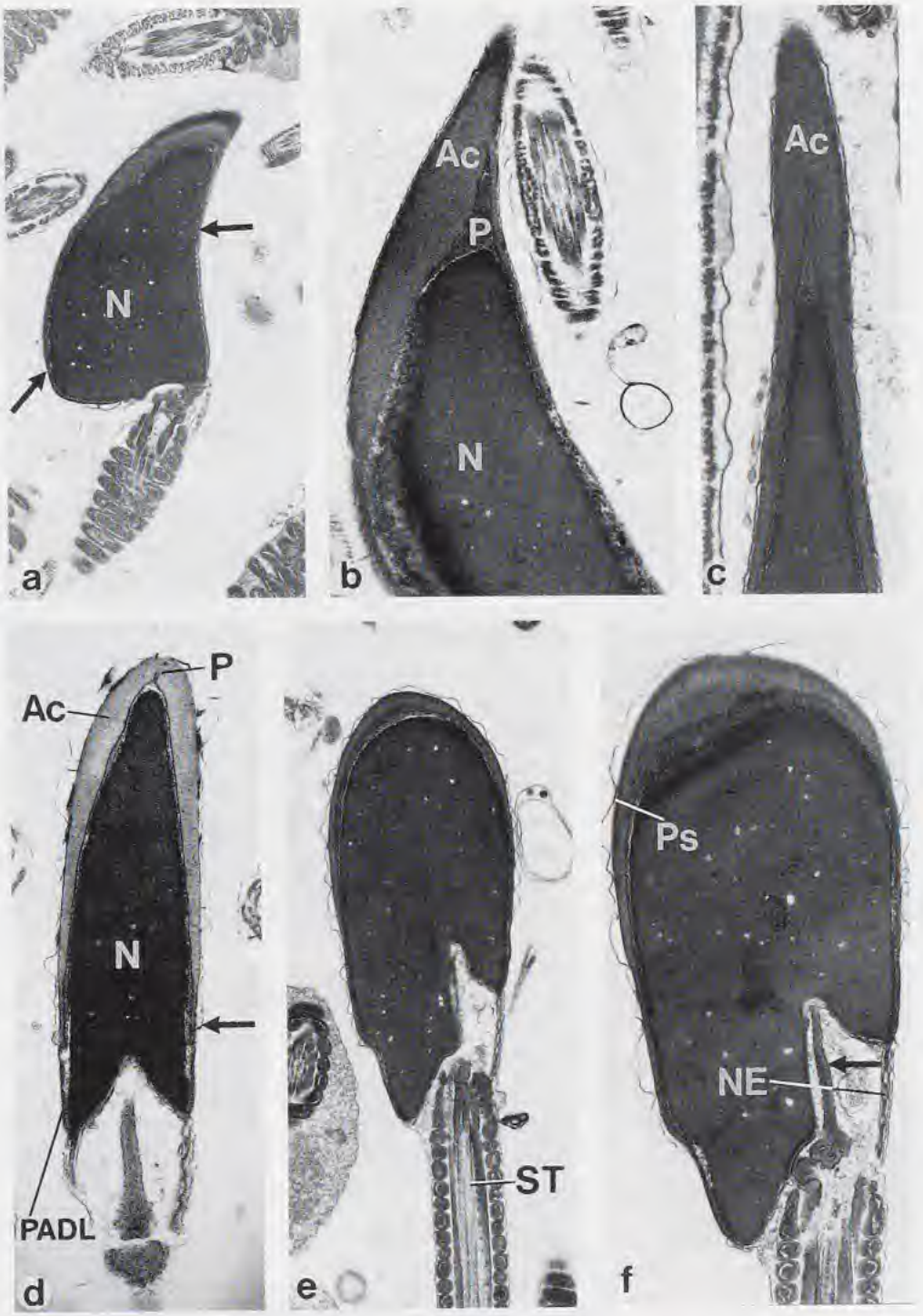


FIG. 2. — *Gerbillurus paeba* and *Tatera leucogaster*. **a-c:** Sperm of *G. paeba*. **a:** Sperm head narrows apically. **b, c:** anterior to the nucleus (N) there is a perforatorium (P) covered by part of the acrosome (Ac). **d-f:** *T. leucogaster*. **d:** spermatozoa have bilaterally flattened nuclei capped by a nearly symmetrical acrosome with small equatorial segment (arrow). **e, f:** connecting piece is unusual in having vertically projecting basal plate and partly bordered by fold of nuclear envelope (NE). a, x 9 100; b, x 15 000; c, x 31 600; d, x 18 200; e, x 11 200; f, x 17 400.

Source : MNHN, Paris

Cricetomyinae

Saccostomus campestris. The sperm head is falciform, has a length of 7 µm, maximum breadth basally of 3 µm, and apical hook, which curves sharply caudad, of about 6 µm (Fig. 3a). The sperm tail connects to the midbasal region, and has a midpiece length of about 25 µm and principal and end piece of about 100 µm.

The acrosome is largely restricted to the convex (Fig. 3b, c) and upper lateral surfaces of the sperm head with the posterior region forming a narrow equatorial segment (Fig. 3e, f). Anteriorly the inner acrosomal membrane is invaginated medially within which electron-dense material of the subacrosomal space occurs (Fig. 3f). Transverse sections of the apical hook show that basally it is bilaterally flattened and contains a nuclear extension which is completely surrounded by the acrosome except midventrally (Fig. 3g). Most of the hook does not contain nuclear material but the acrosome passes along its length and distally becomes triangular in cross section (Fig. 3h, i). In addition, within the apical hook, a small midventral extension of the subacrosomal space occurs which is in contact with the plasmalemma (Fig. 3h, i).

Dendromurinae

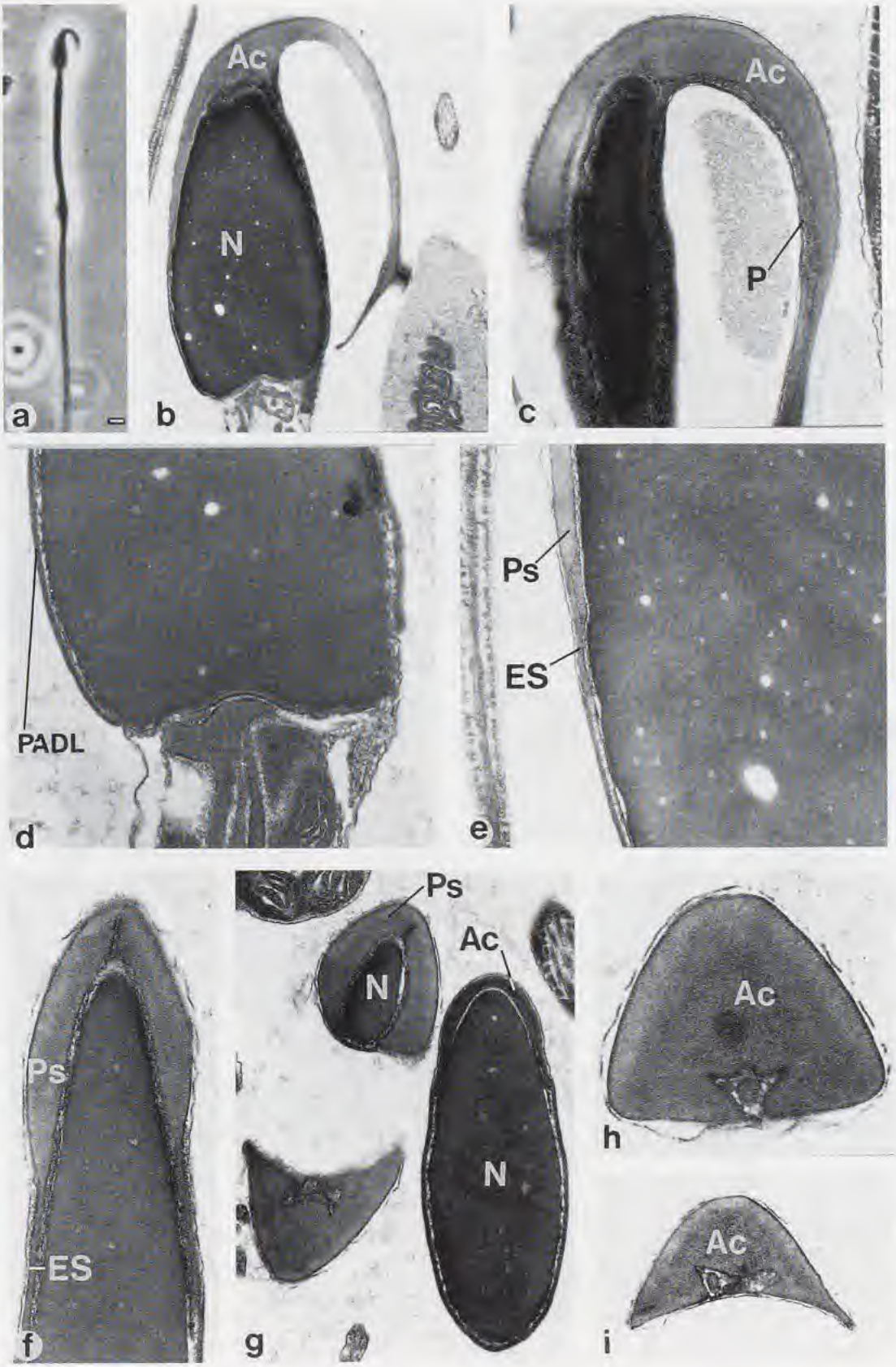
Prionomys batesi, *Steatomys parvus*, *Dendromus mesomelas*, *D. mystacalis*, *Malacothrix typica* and *Deomys ferrugineus*. Only material from *M. typica* was available for TEM. LM indicates significant interspecific differences in sperm head morphology. In *P. batesi* and *S. parvus* a small triangular sperm head is evident that is broadest basally and tapers apically to a hook that is flexed caudad. The connecting piece of the tail attaches midbasally (Fig. 4a, d). Sperm heads of *D. mystacalis* and *D. mesomelas* (Fig. 4b, c) are similar in form, have a lateral face of about 2 µm in diameter, and a 3-5 µm hook that extends from the convex surface and projects caudally, and on the ventral margin a small anteriorly-projecting spike. The sperm tail attaches off-centre basally and is about 90 µm long.

The third sperm type of *Deomys ferrugineus* (Fig. 4e) has a very small, 5 µm, bilaterally flattened head that lacks an apical hook. Anteriorly there appears to be a somewhat ridged, cap-like structure, presumably the acrosomal region, and posteriorly the sperm head tapers a little towards the connecting piece of the sperm tail which has a midpiece of 22 µm and principal and end piece of about 70 µm.

TEM of *Malacothrix typica* sperm shows that its head is largely composed of an electron-dense nucleus which does not appear to extend into the long apical hook (Fig. 4g, h). The acrosome is present along much of the convex surface and makes up most of the material in the apical hook which becomes triangular in cross section. There is a typical subacrosomal space between the inner acrosomal membrane and outer nuclear envelope that extends into the apical hook and is in contact with the plasmalemma midventrally.

FIG. 3. — Sperm of *Saccostomus campestris*. **a, b:** Sperm head is falciform with midbasal attachment of tail. **b, c:** apical hook is long and largely composed of acrosome (Ac). **d:** posterior to the acrosome is the postacrosomal dense lamina (PADL). **e:** acrosome has a very short equatorial segment (ES). **f:** frontal sections show perforatorium extends apically. **g:** TS through apical hook shows nucleus basally distal to which the hook becomes triangular and composed of acrosome except for small perforatorial extension. **h, i:** small perforatorial extension. a, x 900; b, x 11 400; c, x 17 000; d, x 26 600; e, x 33 000; f, x 35 800; g, x 22 700; h, x 50 100; i x 25 300.

Source : MNHN, Paris



Source : MNHN, Paris

Otomyinae

Otomys irroratus. Light microscopy indicates that the sperm head is falciform with the connecting piece of the sperm tail attached on the lower concave surface (Fig. 4f).

TEM shows that a typical bilaterally flattened, electron-dense, nucleus extends into the apical hook which is triangular in cross section. Within the hook the acrosome splits into a dorsal and dorsolateral crest over the convex region and a much smaller, flat, head cap segment close to the ventral margin. Most of the material in the apical hook is composed of the perforatorium that has three prongs, one that is close to the medial convex margin and the other two, much larger ones, which occur ventrolaterally.

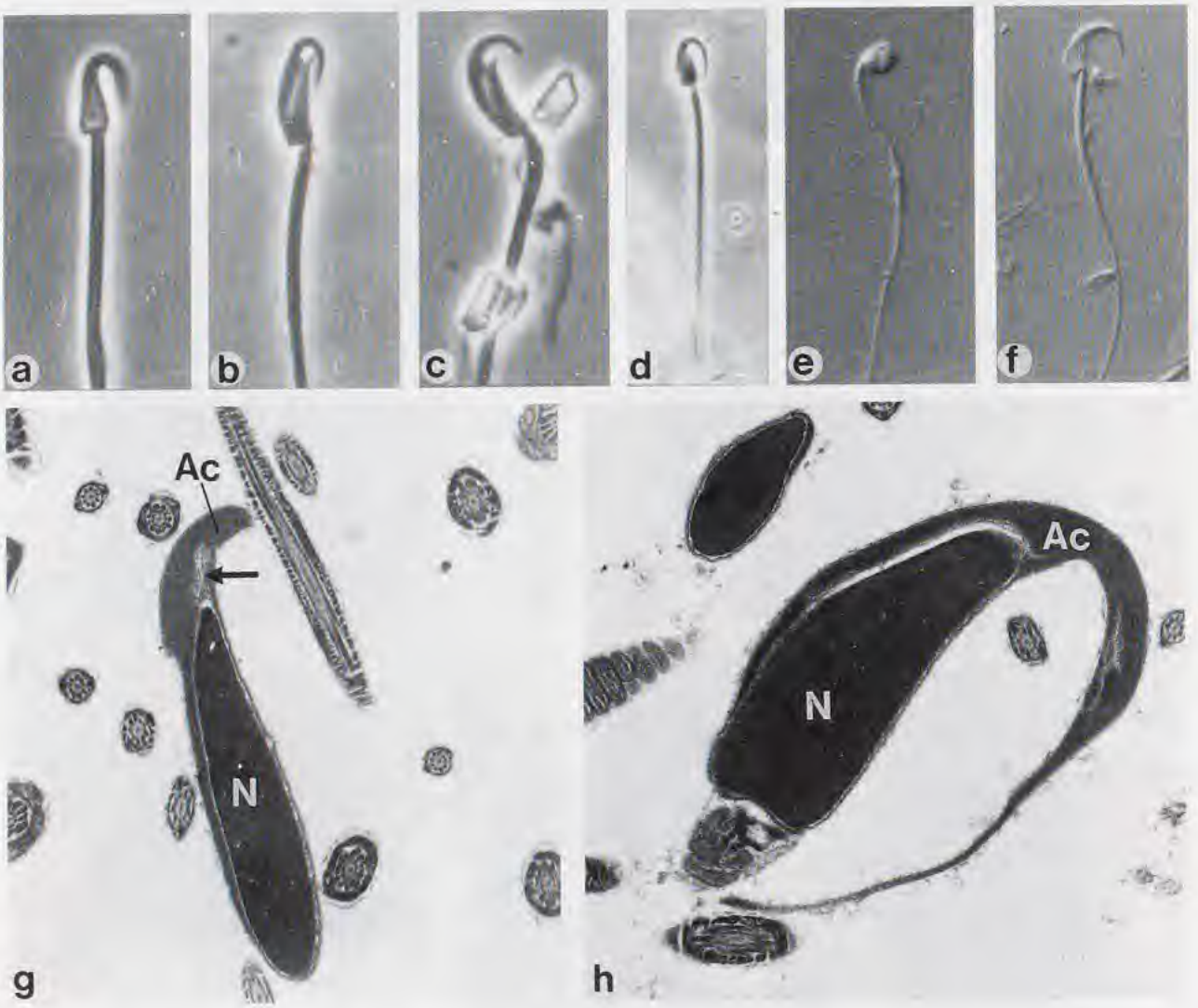


FIG. 4. — Sperm from dendromurine and otomyine species, light microscopy. **a:** *Prionomys batesi*; **b:** *Dendromus mystacalis*; **c:** *Dendromus mesomelas*; **d:** *Steatomys parvus*; **e:** *Deomys ferrugineus*; **f:** *Otomys irroratus*. **g, h:** *Malacothrix typica*, TEM of sperm head. x 700; b, x 1 600; c, x 1 600; d, x 700; e, x 1 100; f, x 600; g, x 9 100; h, x 11 000. a-d, phase contrast; e, f, Nomarski optics.

Source : MNHN, Paris

DISCUSSION

Most eutherians have a spatulate sperm head nucleus that is capped by an acrosome of variable size, but in most murine rodents the sperm head is falciform with the tail attached to the lower concave surface [6-10, 20]; there is a very large and elaborate apical perforatorium which passes back over the anterior nuclear region as three prongs [25, 33]. The sperm head of the golden hamster (a member of the Cricetinae) is also falciform, but its ultrastructure is very different with the acrosome occurring as a large cap over the dorsal and upper lateral nuclear surface, a bilaterally flattened apical hook, and the sperm tail attached to the head midbasally [19, 29, 41]. The ultrastructure of the sperm head of *Mystromys alba* in general resembles that of the golden hamster except that the sperm head is a little narrower and there is a much larger apical acrosomal segment.

In *Saccostomus campestris* the ultrastructural characteristics of the acrosome, perforatorium, and site of attachment of the connecting piece of the sperm tail are somewhat similar to those of the golden hamster and *Mystromys alba* although the perforatorium and acrosome are less extensive apically. This similar internal structural organisation in these three species, two of which come from subfamilies outside the dendromurine-cricetomyine-otomyine-murine clade, suggests very strongly that it represents the ancestral type for this group.

Amongst the dendromurines *Deomys* has sometimes been separated from the others and even placed within its own subfamily [17]. The present data on sperm morphology support the view that *Deomys* is highly divergent, whereas the ultrastructural characteristics of sperm head and site of tail attachment of *Malacothrix typica* are similar to those of *Saccostomus campestris*. The light microscopical observations suggest that *S. parvus* and *P. batesi* sperm are also similar. The ultrastructure of *M. typica* sperm does not resemble that of most of the murines [8, 9, 25] which probably have a derived sperm type (see below). Thus there is no support for close affinity of these groups from these data.

The position of the gerbils (subfamily Gerbillinae) has changed over the years. SIMPSON [37] placed this subfamily within the Cricetidae, but subsequent biochemical data suggested that the Gerbillinae could be the sister-group of the Murinae [13]. The present study indicates that the spermatozoon structure of both *Tatera leucogaster* and *Gerbillurus paeba* is highly derived and quite unlike that of any other genus outside the Gerbillinae; no phylogenetic inferences can thus be drawn.

Over the last 10 years it has become apparent that *Acomys*, previously thought to be a typical murine, may not in fact be part of this large African murid group [36, 40]. It has subsequently been suggested that *Acomys*, *Uranomys* and *Lophuromys* form a clade that is either an early offshoot of the Murinae or closer to the gerbils [13, 15, 16]. The ultrastructure of the sperm acrosome and perforatorium of both *Acomys* and *Uranomys* is more similar to that of *Saccostomus campestris* and the golden hamster than to that of typical murines. However, unlike sperm of *S. campestris*, dendromurines, and cricetines, the sperm tail attaches to the lower concave surface of the sperm head similar to that of the falciform sperm of murine rodents [5]. This trait is thus shared between most members of the Murinae and *Acomys-Uranomys* to the exclusion of the other groups except the Otomyinae [2]. These data support the view that the *Acomys-Uranomys* (and perhaps *Lophuromys*, which has a highly derived sperm type [6]) clade may have branched off from the base of the murine-otomyine radiation [4, 16, 30]. Furthermore, the present study extends the recent observations on the sperm of *Otomys* [2, 5] in confirming that the head is falciform and showing that the structure of the acrosome and perforatorium, as well as the site of attachment of the sperm tail, is identical to that of murines [1, 3, 5-10, 25]. These ultrastructural characteristics of the sperm head do not occur in species from any other subfamily nor do they occur in *Acomys* and *Uranomys*. They are thus likely to be a synapomorphic character shared by most members of the Otomyinae and Murinae to the exclusion of *Acomys* and *Uranomys* and other subfamilies. The findings thus support the previous

Source : MNHN, Paris

suggestion [5] that these two subfamilies are sister-groups and that, in spite of a recent claim to the contrary [35], the falciform sperm head is the ancestral condition within the Murinae.

The following tentative phylogenetic conclusions can be drawn from this study:

1. *Saccostomus* is not part of the Murinae.
2. Within the Dendromurinae, *Deomys* is highly derived and may not be close to the other members of this group.
3. The Dendromurinae is not particularly close to the Murinae.
4. The Otomyinae is the sister-group of the Murinae.
5. The *Acomys-Uranomys* lineage diverged from the base of the Murine-Otomyine clade.

Clearly further insight into the phylogenetic relationships of African muroids could be achieved by carrying out electron microscopy on spermatozoa of more species when, or if, they become available; sperm from more genera of dendromurines and gerbillines need to be investigated. In this study there was no material available from any of the Malagasy rats and mice (subfamily Nesomyinae), maned rats (subfamily Lophiomyinae), or rock and climbing swamp mice (subfamily Petromyscinae). Spermatozoal morphology of these species needs to be investigated before a full appreciation of its significance to the understanding of the evolution of the rats and mice of Africa can be ascertained.

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Source : MNHN, Paris

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Source : MNHN, Paris

Comparative Sperm Structure in Bats (Chiroptera): Some Taxonomic and Adaptive Implications

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ABSTRACT

In Megachiroptera, the spermatozoa of Pteropodidae have a wide spoon-shaped head, a large acrosome, a rounded nucleus and a tail with a long midpiece. In Microchiroptera, the spermatozoa of Rhinolophidae have a spatulate head with a large acrosome, being generally uniform in morphology at the specific level, but the midpiece varies in length with species. In Vespertilionidae examined, two subfamilies of Vespertilioninae and Murininae are separable from another subfamily Miniopterinae in having a small acrosome and long midpiece. Furthermore, in the vespertilionine bats, greater uniformity in sperm structure prevails in the tribe Myotini (*Myotis*) and the tribe Plecotini (*Plecotus* and *Barbastella*) when compared with the tribe Pipistrellini (*Pipistrellus*, *Nyctalus* and *Vespertilio*). The sperm nucleus of the tribe Myotini *s. lat.* (*Myotis*, *Plecotus* and *Barbastella*) is longer than that of Pipistrellini, and the midpiece is obviously distinctive at the generic and specific levels. The midpiece in *Myotis nattereri*, *Myotis macrodactylus* and *Pipistrellus abramus* is longer than that in the other vespertilionine bats examined, and their mitochondria are large in number and in size. The present study demonstrates the potential usefulness of sperm morphology in taxonomic studies of Chiroptera. In addition, the presence of a well-developed midpiece with a unique arrangement of abundant, large mitochondria in spermatozoa of hibernating bats is explained as an adaptive phenomenon for prolonged survival of spermatozoa.

RÉSUMÉ

Structure comparée du spermatozoïde chez les Chauve-souris (Chiroptera): quelques implications pour la taxonomie et l'adaptation

Chez les Megachiroptera, le spermatozoïde des Pteropodidae a une tête large en forme de cuillère, un grand acrosome, un noyau arrondi et une queue avec une pièce intermédiaire longue. Chez les Microchiroptera, le spermatozoïde des Rhinolophidae a une tête spatulée avec un grand acrosome qui est généralement de morphologie uniforme au niveau spécifique, mais la longueur de la pièce intermédiaire varie selon les espèces. Chez les Vespertilionidae qui ont été examinés, les deux sous-familles des Vespertilioninae et Murininae peuvent être distinguées de l'autre sous-famille Miniopterinae grâce à leur petit acrosome et leur longue pièce intermédiaire. De plus, chez les Vespertilioninae, l'uniformité de la structure du spermatozoïde est plus profonde dans les tribus Myotini (*Myotis*) et Plecotini (*Plecotus* et *Barbastella*) que dans la tribu Pipistrellini (*Pipistrellus*, *Nyctalus* and *Vespertilio*). Le noyau du spermatozoïde dans la tribu Myotini *s. lat.* (*Myotis*, *Plecotus* et *Barbastella*) est plus long que celui des Pipistrellini, et la pièce intermédiaire permet de distinguer de manière évidente les genres et les espèces. Chez *Myotis nattereri*, *Myotis macrodactylus* et *Pipistrellus abramus*, la pièce intermédiaire est plus longue que celle des autres Vespertilioninae examinés et les mitochondries sont grandes et nombreuses. Cette étude démontre l'utilité potentielle de la morphologie du spermatozoïde pour les études taxonomiques sur les Chiroptera. De plus, la présence d'une pièce intermédiaire bien développée avec une disposition originale de mitochondries grandes et abondantes chez les chauve-souris qui hibernent est expliquée comme un phénomène adaptatif pour prolonger la survie des spermatozoïdes.

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Source : MNHN, Paris

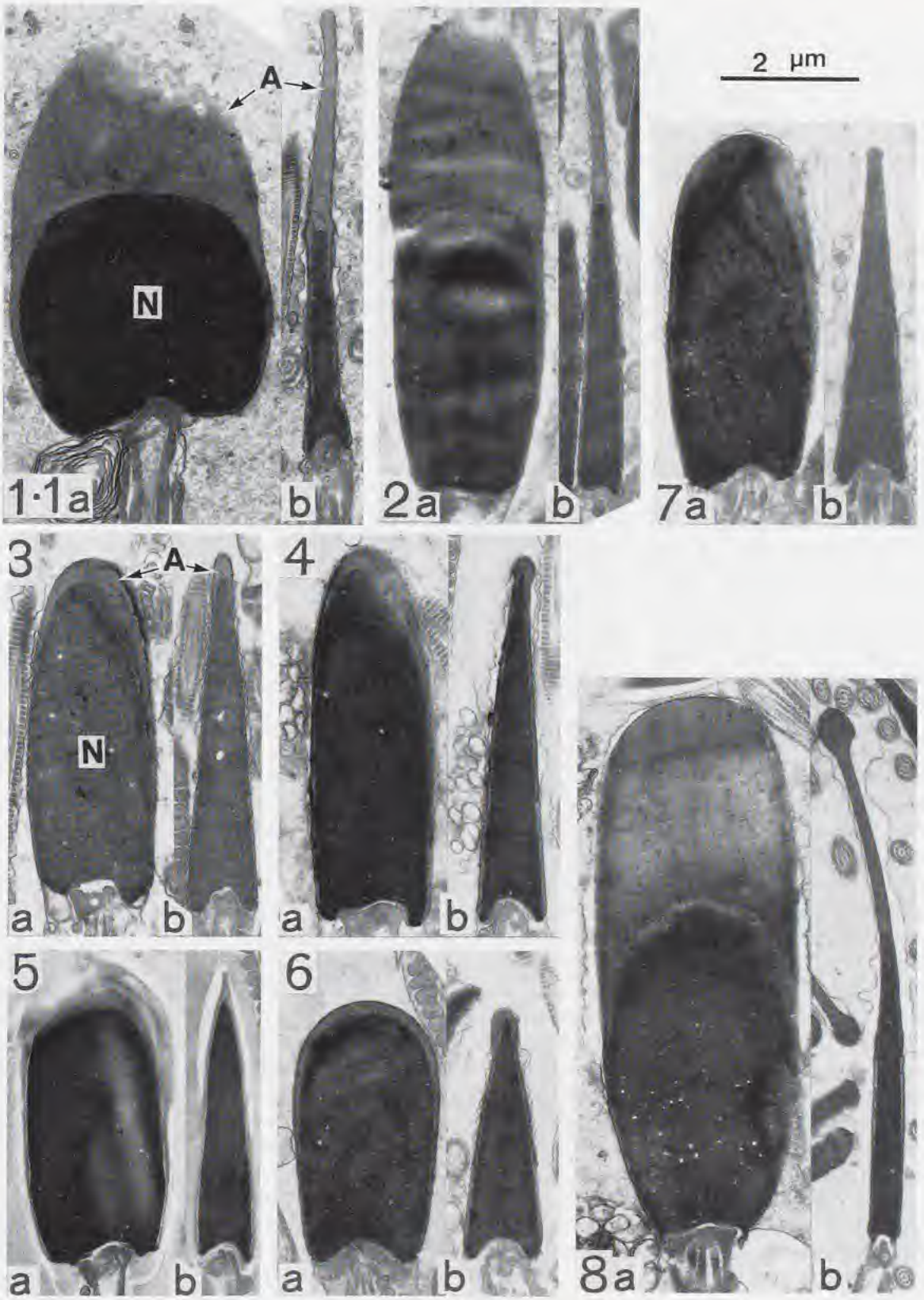
The phylogenetic system must be built up on conclusions obtained from various fields of study. The morphology of spermatozoa is believed to be a good tool for better understanding of phylogenetic relationships, because of their conservatism against environmental factors. In this connection, there have been some light microscopical studies on the morphology of chiropteran spermatozoa [2-5]. On the other hand, electron microscopic studies have been made to analyse sperm survival mechanisms in hibernating bats from the viewpoint of physiological adaptation [12, 17, 19, 22]. The ultrastructure of the sperm of pteropodids has also been described [18]. The aim of the present study is to examine with the electron microscope the comparative morphology of epididymal spermatozoa in 25 bat species (Table 1), and to discuss some taxonomic and adaptive implications of sperm fine structure.

TABLE 1. — Species examined.

Suborder	Family	Subfamily, Tribe	Species	Locality
Megachiroptera	Pteropodidae	Pteropodinae	*Pteropus dasymallus*	Japan
			Cynopterus brachyotis	Malaysia
		Macroglossinae	*Macroglossus minimus*	Malaysia
			Eonycteris spelaea	Malaysia
Microchiroptera	Rhinolophidae	Rhinolophinae	*Rhinolophus cornutus*	Japan
			Rhinolophus monoceros	Taiwan
			Rhinolophus imaizumii	Japan
			Rhinolophus ferrumequinum	Japan
			Rhinolophus luctus	Taiwan
	Vespertilionidae	Vespertilioninae		
		Myotini	*Myotis adversus*	Taiwan
			Myotis nattereri	Japan
			Myotis hosonoi	Japan
			Myotis macrodactylus	Japan
			Myotis formosus	Korea
			Myotis lucifugus	Canada
		Plecotini	*Plecotus auritus*	Japan
			Barbastella leucomelas	Japan
		Pipistrellini	*Pipistrellus endoi*	Japan
			Pipistrellus javanicus	Indonesia
			Pipistrellus abramus	Japan
			Pipistrellus avii	Korea
			Nyctalus noctula	Japan
			Vespertilio superans	Japan
		Murininae	*Murina leucogaster*	Japan
		Miniopterinae	*Miniopterus schreibersii*	Japan

FIG. 1. — Electron micrographs of sperm heads of bat species (a, frontal section; b, sagittal section). The sperm head of Pteropodidae has a spoon-shaped head, a large acrosome (A) and a rounded nucleus (N). The spermatozoa of Rhinolophidae have a spatulate head with a large acrosome. In Vespertilionidae, two subfamilies of Vespertilioninae and Murininae are separable from another subfamily Miniopterinae in having a small acrosome. **1:** *Pteropus dasymallus*; **2:** *Rhinolophus cornutus*; **3:** *Myotis macrodactylus*; **4:** *Barbastella leucomelas*; **5:** *Pipistrellus abramus*; **6:** *Vespertilio superans*; **7:** *Murina leucogaster*; **8:** *Miniopterus schreibersii*.

Source : MNHN, Paris



Source : MNHN, Paris

MATERIAL AND METHODS

The order Chiroptera is divided into two suborders; Megachiroptera, including a single family Pteropodidae, and Microchiroptera, consisting of the remaining families. Bat specimens examined were as follows: four genera and four species representing the family Pteropodidae, one genus and five species of the family Rhinolophidae, and eight genera and sixteen species of the family Vespertilionidae (see Table 1). The cauda epididymidis of each species was fixed in cold 3% glutaraldehyde buffered with 0.1 M-phosphate buffer (PBS) at pH 7.4, then rinsed thoroughly with the same PBS and postfixed in cold 1.3% osmium tetroxide in the PBS, dehydrated with alcohol and embedded in Epon 812 (Taab). Sections (60 nm) were doubly stained with uranyl acetate and lead citrate before examination in an Hitachi HS-9 or an Hitachi H-600A electron microscope (75 kV).

OBSERVATIONS

In Megachiroptera, the *Pteropus dasymallus* spermatozoon, when cut frontally, has a dorsoventrally flattened, wide spoon-shaped head with convex lateral contours. The head is 6 μm in length, of which the posterior 3.2 μm is occupied by the rounded nucleus, being about 3.4 μm in width (Fig. I 1a). When cut sagittally, the nucleus has a convexity in the posterior region, and the acrosome closely fits into an anterior concavity; thus, the nucleus appears rocket-shaped, being thicker at the base, and tapering to the tip (Fig. I 1b). The acrosome protrudes for a length of 2.8 μm from the tip of the nucleus. The equatorial segment of the acrosome occupies the posterior one-fourth of the nucleus. Thus, the postacrosomal region is shorter than that in Microchiroptera (cf. Fig. I 2-8). The inner acrosomal membrane is serrated, while it is smooth in Microchiroptera. The sperm tail is very long, because of the conspicuously long midpiece with a length of 22.0 μm (Fig. II 1a, b). Mitochondria are large in number, but small in size (Fig. II 1a, 3a). The arrangement of mitochondria in *Pteropus* is similar to that in common mammalian species, in which mitochondria are disposed in a helix with their tapering ends overlapping or abutting end-to-end. The characteristics of the *Pteropus dasymallus* spermatozoon resemble those of the other three genera examined in Pteropodidae (Fig. II 1b).

In Rhinolophidae, of the Microchiroptera, the spermatozoon of *Rhinolophus cornutus* has a dorsoventrally flattened, spatulate head with parallel sides and a narrow nucleus. The nucleus occupies 4.1 μm of the head with a total length of 6.8 μm, being about 2.1 μm in width (Fig. I 2a). When cut sagittally, the nucleus appears extremely slender and wedge-shaped, being thicker at the base, and tapering to a point (Fig. I 2b). The large acrosome extends anteriorly beyond the leading edge of the nucleus for a length of 2.7 μm. The equatorial segment of the acrosome is situated at about the middle portion of the nucleus (Fig. I 2b). Thus, the postacrosomal region is longer than that in Megachiroptera. The midpiece (12.0 μm) in this species is very short compared with the midpiece (15.0 μm) with abundant mitochondria in *R. ferrumequinum* (Fig. II 2a, d). In *Rhinolophus*, the sperm head is generally uniform in morphology at the specific level, but the midpiece varies in length with the species (Fig. II 2a-e).

In Vespertilionidae, two subfamilies of Vespertilioninae and Murininae are separable from the subfamily Miniopterinae in having a small acrosome and long midpiece. Furthermore, in the vespertilionine bats examined, greater uniformity in sperm structure prevails in the tribe Myotini (*Myotis*) and the tribe Plecotini (*Plecotus* and *Barbastella*) when compared with the tribe Pipistrellini (*Pipistrellus*, *Nyctalus* and *Vespertilio*). The sperm nucleus of the tribe Myotini *s. lat.* (*Myotis*, *Plecotus* and *Barbastella*) is longer than that of Pipistrellini (Fig. I 3-6). For example, the sperm nucleus occupies 4.9 μm of the head (5.1 μm in length) in *Myotis macrodactylus*, and 3.7 μm of the head (4.0 μm in length) in *Pipistrellus abramus*. At the specific level, the spermatozoa of *Pipistrellus endoi* and *P. javanicus* have a narrower head and shorter midpiece than the *P. abramus* spermatozoon.

In Vespertilionidae, the midpiece is clearly distinctive at the generic and specific levels. The midpiece in *Myotis nattereri*, *Myotis macrodactylus* and *P. abramus* is longer than that in the other vespertilionine bats examined, and their mitochondria are large in number and in size (Fig. II 3-6). The arrangement of mitochondria is such that each turn of the flat helix is made up of just two

Source : MNHN, Paris

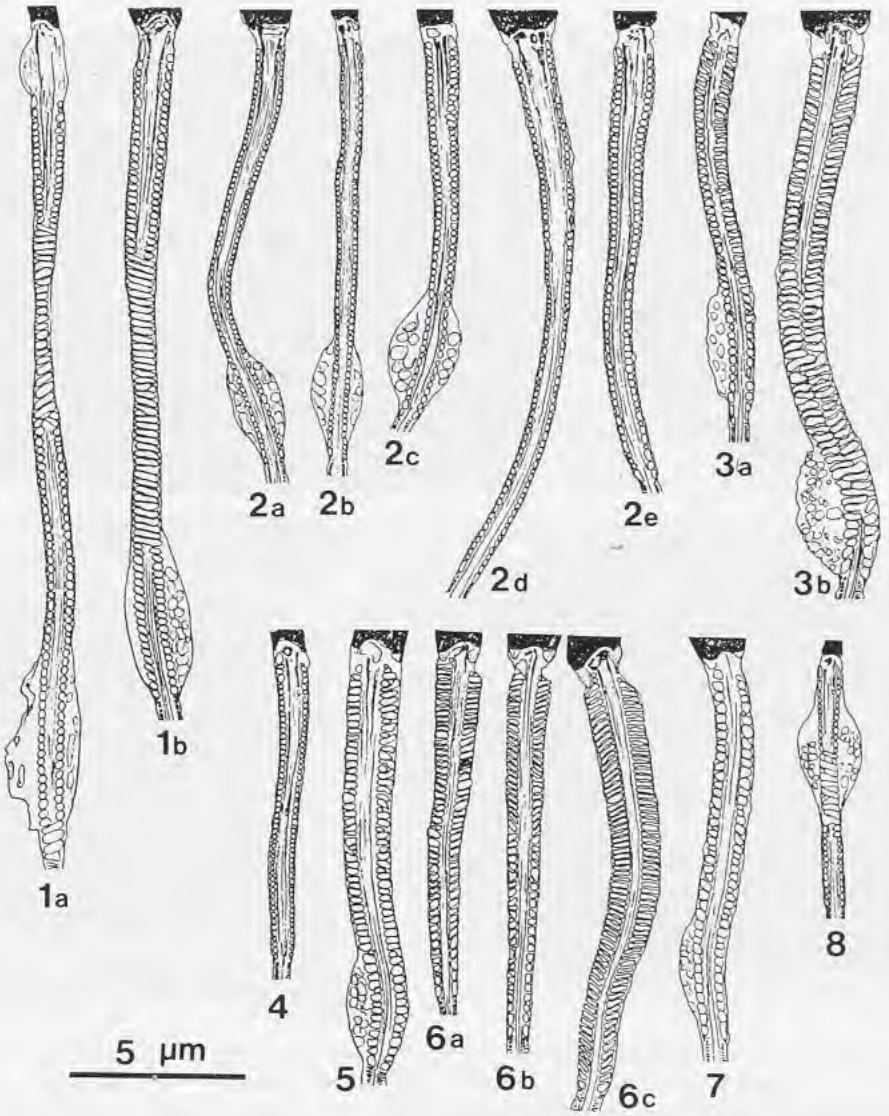


FIG. II. — Semischematic reconstruction of the midpiece in bats, based on electron microscopic observations. **1a:** *Pteropus dasymallus*; **1b:** *Macroglossus minimus*; **2a:** *Rhinolophus cornutus*; **2b:** *Rhinolophus monoceros*; **2c:** *Rhinolophus imaizumii*; **2d:** *Rhinolophus ferrumequinum*; **2e:** *Rhinolophus luctus*; **3a:** *Myotis hosonoi*; **3b:** *Myotis nattereri*; **4:** *Plecotus auritus*; **5:** *Barbastella leucomelas*; **6a:** *Pipistrellus endoi*; **6b:** *Pipistrellus javanicus*; **6c:** *Pipistrellus abramus*; **7:** *Murina leucogaster*; **8:** *Miniopterus schreibersii*.

large mitochondria whose end-to-end junctions are directly opposite on the dorsal and ventral aspects of the midpiece (Fig. III b, c). The two opposite mitochondria are conspicuously thick and ovoidal in *M. nattereri* and *M. macrodactylus* (Fig. III b), and rhombiform in *P. abramus* (Fig. III c).

In *Murina leucogaster*, in the Murininae, the sperm nucleus occupies 4.0 µm of the spatulate head (4.7 µm in length), and is about 2.0 µm in width (Fig. I 7a), being wedge-shaped in sagittal section and surmounted by a small acrosome (Fig. I 7b). The spermatozoon of this species resembles that of *Myotis* in morphology and in size. In *Miniopterus schreibersii*, in the

Source : MNHN, Paris

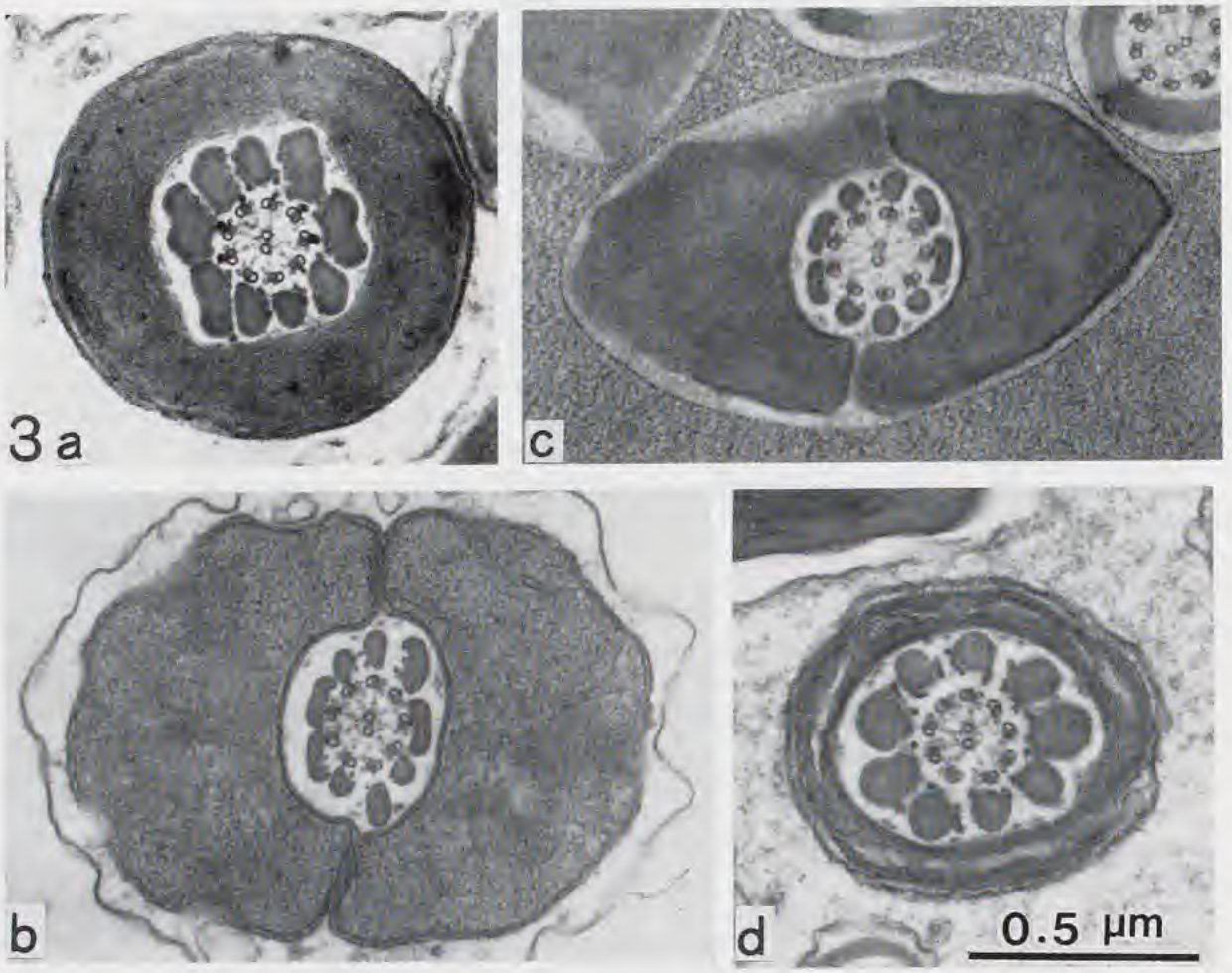


FIG. III. — Electron micrographs of the midpiece (transverse section). **a:** *Pteropus dasymallus*; **b:** *Myotis nattereri*; **c:** *Pipistrellus abramus*; **d:** *Miniopterus schreibersii*. In *Myotis nattereri* and *Pipistrellus abramus*, there are just two large mitochondria to each turn of the sheath: the end-to-end junctions of mitochondria are in line along the dorsal and ventral aspects of the midpiece.

Miniopterinae, the spermatozoon has a large, spatulate head. The nucleus occupies 5.0 µm of the head (8.0 µm in length), being about 2.8 µm in width (Fig.I 8a). When cut sagittally, the head is lanceolate, tapering to a tip (Fig. I 8b). The large acrosome, tipped with a swelling, protrudes anteriorly beyond the leading edge of the nucleus for a length of 3.0 µm. The midpiece in this species is very short compared with that in the other subfamilies examined, and mitochondria are conspicuously small in number and in size (Figs. II 8, 3d).

DISCUSSION

The spermatozoa of the two subfamilies examined, Pteropodinae and Macroglossinae in the Pteropodidae, belonging to the Megachiroptera, are similar in possessing a flattened, wide spoon-shaped head with convex lateral contours, a large acrosome, a rounded nucleus and a tail with a long midpiece. The fact that Pteropodidae share such similarities in sperm morphology with Soricidae which is a group of the basic insectivores retaining primitive characters within

Source : MNHN, Paris

eutherians, strongly supported MILLER's concept [11] that Megachiroptera should be allocated to the lowest taxon within the Chiroptera from the viewpoint of adaptation for flight.

In Microchiroptera, the spermatozoon of *Emballonura furax*, belonging to the Emballonuridae, has a spatulate head according to the figure presented [6], although the authors do not refer to such a morphological character. The spermatozoa of Phyllostomatidae have a broad head as well as those of Megachiroptera, and are reasonably constant at the generic and specific levels [3], differing from those of Rhinolophidae and Vespertilionidae in morphology. In *Rhinolophus*, the sperm head, with a large acrosome. is uniform in morphology at the specific level. In *R. ferrumequinum*, the midpiece, with abundant mitochondria, is very long compared with the other four species examined. This species copulates in autumn, and the spermatozoa are stored in the female reproductive tract until the next spring (sperm storage type) [19]. Thus, the well-developed midpiece in *R. ferrumequinum* may be an adaptation for prolonged survival of the spermatozoa. The reason why the midpiece in the other four species of *Rhinolophus* is shorter than that in *R. ferrumequinum* is unknown as their reproductive patterns have not yet been examined in detail.

It has been suspected on the basis of morphological characters that the vespertilionid ancestor was a *Myotis*-like bat. According to ANDO [1], the karyotypes of *Myotis* are characterized by having a prototypic karyotype for the Vespertilionidae, and the karyotypes of Myotini and Plecotini (Myotini *s. lat.*), Pipistrellini and Nycticeini seem to be derived from the ancestral karyotype of the Vespertilionidae. As to *Myotis*, the prototypic karyotype is said to have remained unchanged at or even after divergence, and consequently their karyotypes resemble each other at the specific level. Also from sperm morphology, the spermatozoa of *Myotis* are similar in general structure at the specific level, showing a typical structure in Vespertilionidae in possessing a spatulate head and small acrosome. The morphology of the sperm heads is consistent in Myotini (*s. lat.*), thus being in good agreement with the phylogenetic relationships. However, there are great differences in number and in size of mitochondria; for example, the midpiece in *Myotis macrodactylus*, *M. nattereri* and *M. lucifugus* is very long, and their mitochondria are large in number and in size compared with the other three species of *Myotis*. It seems to be an adaptive phenomenon for prolonged sperm survival that the well-developed midpiece is present in *M. lucifugus* in which clear evidence has been obtained for the prolonged fertilizing life of the spermatozoon [21].

In Pipistrellini, taking distinct differences with regard to the karyotype and the baculum morphology between *Pipistrellus abramus abramus* and *P. javanicus javanicus* into consideration, they should be separated as a good species from each other, although they have been classified at a subspecific level, *P. a. abramus* and *P. a. javanicus* [1]. This assumption of specific distinctness is supported also from differences in fine structure of spermatozoa, particularly in number and in size of mitochondria. Further, the well-developed mitochondrial sheath in *P. pipistrellus* [16, 17] and *P. abramus* [7, 8, 20] with unusual reproductive habits (sperm storage type) may be concerned with an adaptation for prolonged survival of spermatozoa, as in *M. lucifugus* mentioned above. The sperm nucleus of Pipistrellini (*Pipistrellus*, *Nyctalus* and *Vespertilio*) is shorter than that of Myotini (*s. lat.*), and the above three genera are consistent in sperm morphology. As for Murininae, the subfamily has been regarded as a conservative taxon with primitive characters, being Myotini-like in sperm morphology.

In *Miniopterus schreibersii*, in the Miniopterinae, copulation is followed closely by ovulation in mid-October [12-15]. Embryonic development, however, proceeds very slowly during the long hibernation period (delayed implantation type) [9]. Correspondingly, the *M. schreibersii* spermatozoon differ conspicuously from the other subfamilies examined (Vespertilioninae, Murininae) in having a spatulate head surmounted by the remarkably large acrosome, and a very short midpiece whose mitochondria are conspicuously small in number and in size, compared with the other subfamilies. Such great differences in sperm morphology clearly reveal subfamilial distinctness. On the basis of the dental formula [10], it has been suggested that

Source : MNHN, Paris

the genus *Miniopterus* be placed in a separate family Miniopteridae. The fact that the spermatozoon has the very short midpiece, mentioned above, may be associated with the fact that the spermatozoon does not need to retain its viability for as long as 5-7 months unlike the sperm storage type; thus, *M. schreibersii* is in a striking contrast to bats belonging to the sperm storage type with a well-developed midpiece.

In conclusion, the present study has demonstrated the potential usefulness of sperm morphology in taxonomic studies of Chiroptera. The presence of a well-developed midpiece (unique arrangement of abundant, large mitochondria) of spermatozoa in hibernating bats is explained as an adaptive phenomenon for prolonged survival of spermatozoa, and the details of the reproductive pattern in bats yet to be examined are needed to analyse adaptive implications of sperm morphology.

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Source : MNHN, Paris

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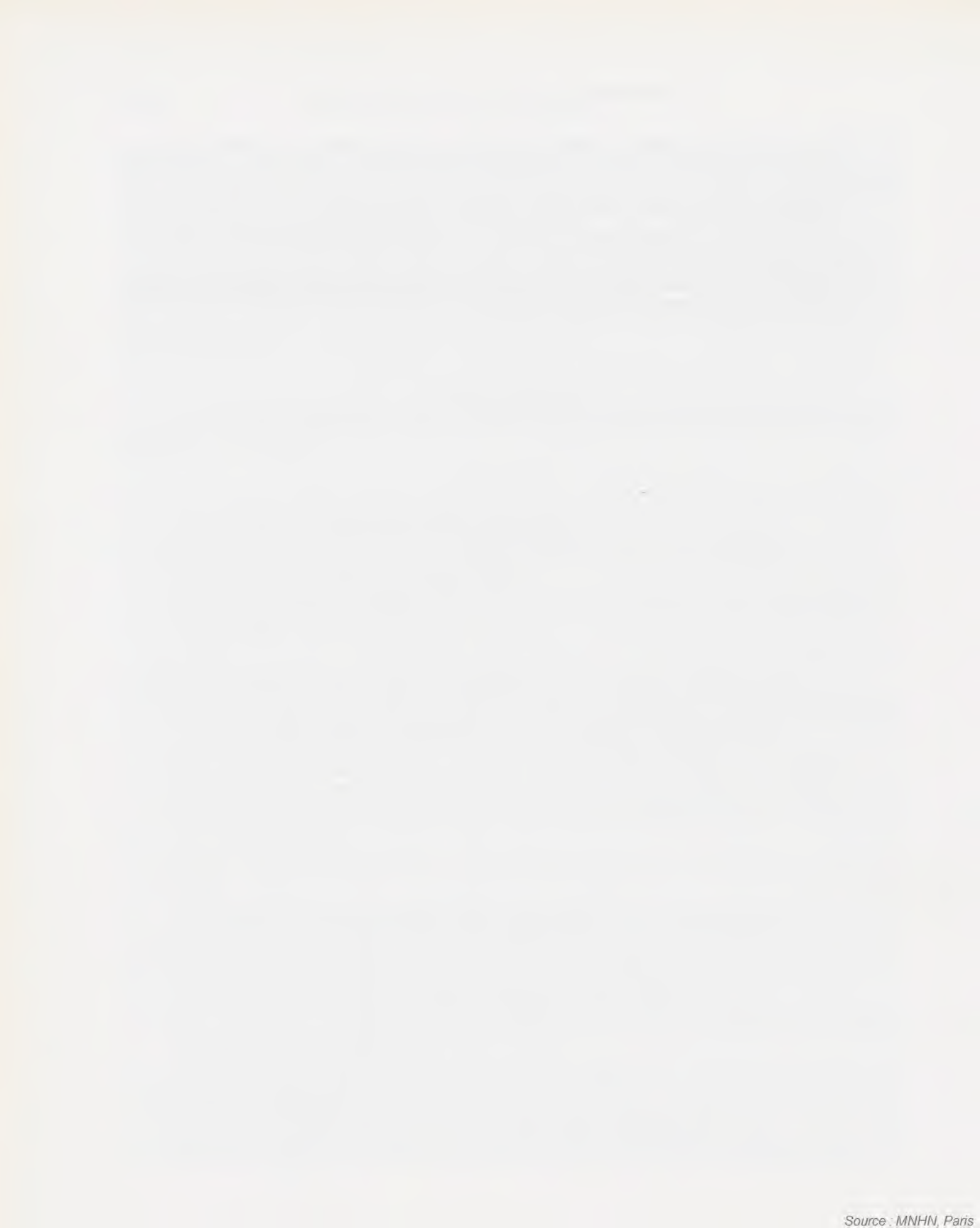
Source : MNHN, Paris



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Molecular and Ontogenic Analysis of the Human Sperm Tail Fibrous Sheath

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ABSTRACT

In the past few years, a considerable amount of data has been obtained regarding the biochemical and antigenic structure of the human sperm tail fibrous sheath (FS). The development of a method for the FS isolation enabled its biochemical characterization, and several polypeptides with MW ranging between 25 and 97 kDa were identified. These were antigenic, and their dissection with monoclonal antibodies (MoAbs) showed the presence of bioactive groups e.g. phosphates and sugars. The bioactive groups were incorporated into the proteins late in spermatogenesis, following the FS assembly, as additional steps of post-translational modifications; this ensures structural maturity of the sperm tails prior to the sperm release to the epididymis. This chapter is a description of these results and their significance.

RÉSUMÉ

Analyse moléculaire et ontogénétique de la gaine fibreuse de la queue du spermatozoïde humain

Au cours des dernières années, une quantité considérable d'informations a été obtenue à propos de la structure biochimique et antigénique de la gaine fibreuse de la queue du spermatozoïde humain (GF). Le développement d'une méthode d'isolation de la gaine fibreuse a permis sa caractérisation biochimique, et plusieurs polypeptides, de poids moléculaire entre 25 et 97 kDa ont été identifiés. Ces polypeptides sont antigéniques, et leur analyse grâce à des anticorps monoclonaux a montré la présence de groupes biologiquement actifs tels que des phosphates et des sucres. Les groupes biologiquement actifs sont incorporés dans les protéines à un stade tardif de la spermatogenèse, après l'assemblage de la GF, grâce à des étapes additionnelles de modifications post-traductionnelles. Ceci garantit la maturité structurale de la queue du spermatozoïde avant la libération du sperme dans l'épididyme. Ce chapitre décrit ces résultats et leur signification.

The fibrous sheath (FS) is a unique cytoskeletal structure of the sperm tail which occupies the principal piece. Its two longitudinal columns run dorso-ventrally along the outer dense fibres numbers 3 and 8, and terminate by attaching to axonemal microtubular doublets numbers 3 and 8. These columns are interconnected by large numbers of transverse ribs [9]. During spermiogenesis, the FS assembly of the human spermatozoon begins at the fourth Sc stage spermatid by the formation of a network of filamentous tubules [8] which are later obliterated through the deposition of electron dense granular material.

Until recently, nothing was known of the molecular or antigenic structure of the human sperm tail FS despite its involvement in various flagellar abnormalities which are associated with sperm immotility and infertility [6, 18, 19, 21]. Molecular studies, therefore, are important to

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understand the biological significance of the FS in sperm motility and to unravel the molecular basis for its anomalous formation. Furthermore, an understanding of the protein synthesis and assembly could lead to the development of new methods of contraception through identifying reagents capable of disrupting these processes without affecting other tissues. Therefore, in the past few years, the main interest of the author was focused on this subject, and the results of these studies are described below.

RESULTS AND DISCUSSION

Biochemical and antigenic analysis of the FS

The development of a method for isolating highly purified human FS preparations enabled biochemical analysis of the FS [22]. As in rodents [5, 10, 26], the SDS-PAGE of human preparations revealed several major protein bands (Fig. 1) with MW ranging between 25 and 97 kDa [22]. The presence of several polypeptide bands is a reflection of the FS structural complexity, although some of these peptides could be degradation products. It is possible that different proteins are utilized in the construction of the various FS components such as the transverse ribs and longitudinal columns, which develop independently [13] and are made up of different sized tubules [9, 28], and the electron dense material deposited in these filaments. In this respect, the FS is similar to neurofilaments whose structure involves the use of three different but closely related polypeptides which are encoded by three genes [30]. However, amino acid sequencing of the FS proteins is essential to determine any sequence homology.

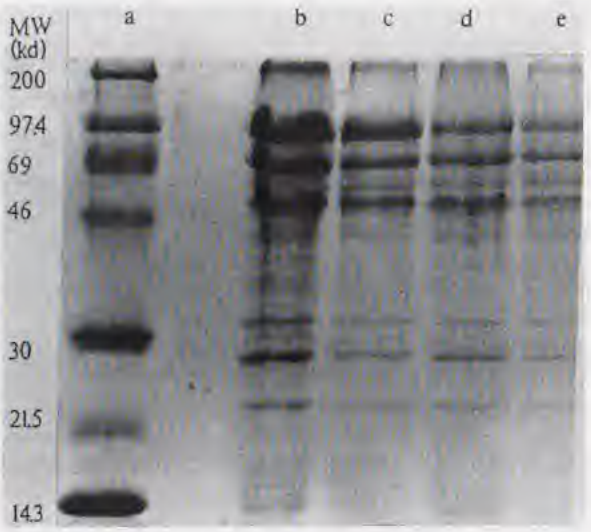

FIG 1. — SDS-PAGE of purified human sperm fibrous sheath (FS) obtained from four normozoospermic donors showing several protein bands, lanes (b-e); a, MW markers. From [22], reproduced by permission of *Human Reproduction*.

The FS polypeptides are highly immunogenic and express antigenic epitopes which are not shared by several somatic tissues. This was demonstrated by using mouse xenoantisera (MAFA) which were raised against purified human sperm FS and stained seven major protein bands (Fig. 2) with MW ranging between 25 kDa and 97.4 kDa by Western blotting [23]. In humans, MAFA did not react with other sperm tail cytoskeletal elements, but in rats, a cross-reaction between the FS and outer dense fibre proteins was reported [26].

In addition to xenoantisera, MoAbs have also been used to investigate the FS antigens. AJ-p97 was the first polypeptide to be identified using RT97 MoAb [14] which was later found to be phosphorylated [20]. In rats, the 80 kDa phosphoprotein reported by amino acid analysis [5] could be analogous to AJ-p97, although amino acid sequencing is required. Other MoAbs included GDA-J/F3 which detected a non-collagenous asialo-glycoprotein [16], AJ-FS1 and AJ-

Source : MNHN, Paris

FS9 MoAbs which recognized several FS-specific antigens [15, 17]. However, unlike other MoAbs, the AJ-FS9 detected masked antigens which required proteolytic enzymes treatment for their exposition; this indicates that the FS assembly involves the deposition of proteins mutilayers [15]. In Western blotting, the reaction of AJ-FS1 and AJ-FS9 with multiple protein bands did suggest the presence of shared epitopes in these polypeptides and possibly some structural homology. Another group of non-glycosylated human FS antigens has recently been described by others using MoAbs [3]. The alpha-spectrin [31] and actin previously identified in the human sperm tail principal piece [11] are more likely to be involved in the subcytoplasmic membrane cytoskeleton [4] than the FS structure.

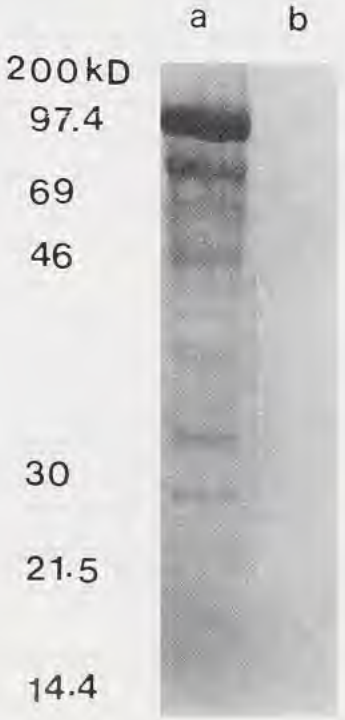

FIG. 2. — **a:** Western blotting and immunostaining of purified FS with MAFA xenoantisera; **b:** negative control. From [23], reproduced by permission of *Journal of Reproductive Immunology*.

During the immunogold EM investigations to localize the target antigens for GDA-J/F3, RT97, AJ-FS1, AJ-FS9 and MAFA, the gold labelling was seen to be restricted to the outer FS surface, i.e. the inner FS surface was not stained [14, 15, 17, 19, 23]. This antigenic polarity of the FS surfaces is determined by the locality of the antigenic epitopes which may incorporate bioactive groups e.g. phosphates or sugars [20, 16]. The presence of these groups together with the intimate association of the FS to the cytoplasmic membranes [24] may indicate some molecular interaction between these two structures during sperm motility.

Ontogeny and post-translational modifications of the FS antigens

The monoclonal and polyclonal anti-FS antibodies were useful for assessing the ontogeny of their target antigens during spermatogenesis. Thus, the restricted reaction of the GDA-J/F3, RT97, AJ-FS1, AJ-FS9 and MAFA with the mature sperm tails and lack of staining of the earlier germ cell stages indicated the late expression of the antigenic epitopes which followed the FS assembly [14, 15, 17, 19, 23]. These data are in contrast to those reported in mice [29] and rats [7, 10, 27]. In mice, for instance, the target antigen for K32 MoAb was first detected in the cytoplasmic matrix of stage 14 and 15 spermatids which then became localized to the FS at stage

Source : MNHN, Paris

16 [29]. This discrepancy could be due to the degree of maturation of the cells used in immunization; the epididymal and/or testicular germ cells preparations of rodents are likely to contain cells which are less mature than the ejaculated human sperm samples.

Following the human FS structural assembly, some of its proteins undergo post-translational modifications including phosphorylation [20], glycosylation [16] and disulphide bonding [2]. The phosphorylation and glycosylation were demonstrated by using RT97 and GDA-J/F3 MoAbs which recognized phosphorylated and glycosylated antigens respectively. These two processes take place in the testis prior to the release of spermatozoa to the epididymis and continue thereafter, whereas the disulphide linking of the FS protein occurs in the epididymis [2]. Although the biological importance of these post-translational modifications is yet to be established, their late occurrence following the FS assembly indicates their involvement in the FS molecular maturation to ensure a functional structure. Phosphorylation of the AJ-p97, for instance, is thought to contribute to the elasticity of the FS through the creation of electrostatic repulsion between the negatively-charged phosphate groups [20], whereas the FS rigidity could be due to the S-S bonding of the FS proteins [2].

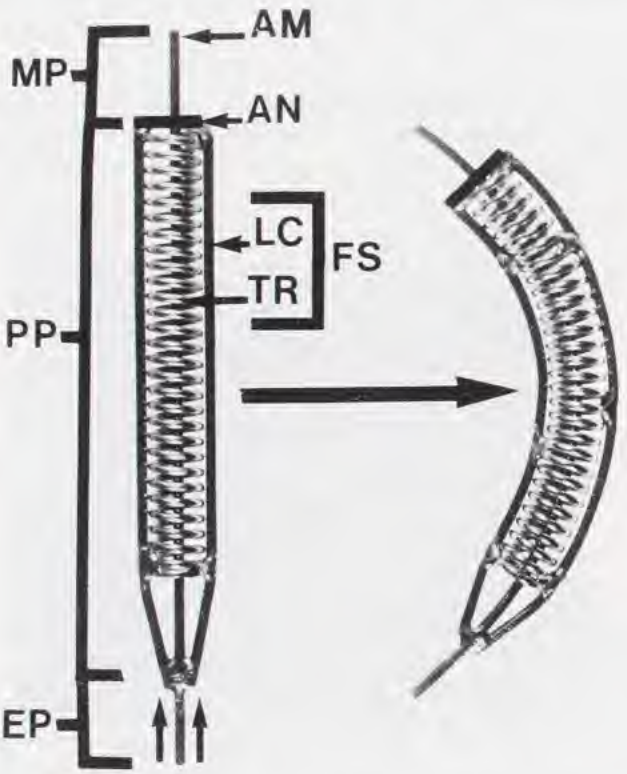

FIG. 3. — Illustration of the FS function. The attachment of the longitudinal columns (LC) to the axonemal microtubules (AM) ensures transmission of the microtubule linear sliding thrust to the FS which bends due to its spring-like features and produces a wave. AN, Annulus; EP, PP, and MP, end-, principal- and middle- piece of sperm tail; TR, Transverse ribs.

The biological function of the FS and its analogy to intermediate filaments

Because of their failure to react with anti-keratin, -vimentin and -neurofilaments antibodies, the human germ cells were previously reported to lack intermediate filaments [1, 12, 25]. However, despite the lack of the conventional antigenic determinants of intermediate filaments, the FS still shares several features with these cytoskeletal elements including: tissue specificity of its antigens [14-17], disulphide cross-linking [2], phosphorylation [20], and insolubility in various detergents and chemicals [22]. Furthermore, the FS develop as filamentous structures although their lumens are obliterated during development [8]. The diameters of the FS transverse rib and longitudinal column tubules are 5-6 nm [28] and 15-20 nm respectively [9], whereas that

Source : MNHN, Paris

of the somatic intermediate filaments is 10-15 nm [30]. All these features indicate that the FS could represent a modified form of intermediate filament. The AJ-p97 which shares a number of features with the neurofilaments including phosphorylation could, therefore, be the FS intermediate filament protein [14, 20]. However, amino acid and/or DNA sequencing is essential to establish its relationship to other intermediate filaments.

Although sperm motility is attributed to the axonemal microtubules, the presence of an additional structure is essential to convert the nonprogressive microtubular sliding into wavy movement. This structure is likely to be the FS due to its anatomical and structural properties. The FS is characterized by its spring-like morphology, rigidity and flexibility, and its location in a confined space; its anterior end starts at the annulus whereas its two longitudinal columns terminate posteriorly by attaching to microtubule doublets 3 and 8. This direct attachment to the microtubules ensures the transmission of the microtubular sliding thrust to the FS, and because of its presence in a confined space and spring-like features, the FS is likely to bend; consequently, the linear movements of the microtubules are converted into waves (Fig. 3). During bending, the proximation of the negatively-charged groups, e.g. the phosphates of AJ-p97, might ultimately lead to electrostatic repulsion and reversal of the original act [20]. In ciliated epithelium, the lack of the FS could be the reason for the inability of cilia to produce waves despite the presence of other axonemal structures.

Current investigations

The lambda gt11 human testicular cDNA libraries have recently been screened with AJ-FS1 MoAb, and two clones identified; their DNA sequence is currently being investigated (unpublished data). This is essential to assess any DNA sequence homology with other cytoskeletal proteins within the sperm tail and somatic cells. Future transfection of suitable host cells with the encoding genes could provide some clues to the synthesis and assembly of the FS proteins. Similar work of amino acid and/or DNA sequencing should be carried out in other species to evaluate their phylogenetic relationship.

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Source : MNHN, Paris

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Source : MNHN, Paris

Diversity of Avian Spermatozoa Ultrastructure with Emphasis on the Members of the Order Passeriformes

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ABSTRACT

The spermatozoa of passerine birds have ultrastructural features which are common to the sperm of members of the other orders of the class Aves. The sperm are filiform, the acrosome is conical in shape, and the nucleus is about the same diameter as the acrosome and midpiece. The helical shape of the passerine sperm, the presence of a helical membrane extending laterally from the acrosome, the 9 peripheral dense fibres (accessory fibres) which extend from a juxtanuclear body along the doublets of the axoneme, and the presence of a single elongated helical mitochondrion are synapomorphies of the passerines. Since avian spermatozoa exhibit considerable variation in gross morphology and in ultrastructure, these variations may be useful indicators of phylogenetic relationships. It is necessary to have details of the fine structure in order to develop a tree of these taxonomic relationships. The Class Aves is divided into a number of orders based on a number of features including morphology, and behaviour. The order Passeriformes contains 5712 (59%) of the 9672 species of recent birds. Most of the passerine birds are small land dwelling birds that tend to exhibit similar morphological features thus making their subdivision into categories below suborders difficult. This study compares some of the ultrastructural differences in sperm of selected members of the order Passeriformes.

RÉSUMÉ

Diversité de l'ultrastructure des spermatozoïdes d'Oiseaux, en particulier des Passeriformes

Les spermatozoïdes des Oiseaux Passeriformes ont des caractéristiques ultrastructurales qui sont communes aux spermatozoïdes des membres des autres ordres de la classe Aves. Les spermatozoïdes sont filiformes, l'acrosome est conique et le noyau est approximativement du même diamètre que l'acrosome et la pièce intermédiaire. La forme hélicoïdale du spermatozoïde, la présence d'une membrane hélicoïdale qui s'étend latéralement à partir de l'acrosome, les 9 fibres denses périphériques (fibres accessoires) qui partent d'un corps juxtanucléaire le long des doublets de l'axonème, et la présence d'une mitochondrie unique, allongée et hélicoïdale sont des synapomorphies des Passeriformes. Comme les spermatozoïdes des Oiseaux montrent des variations considérables de morphologie générale et d'ultrastructure, ces variations peuvent être d'utiles indicateurs des relations phylogéniques. Il est nécessaire de connaître les détails de l'ultrastructure pour créer un arbre des relations taxonomiques. La classe Aves est divisée en plusieurs ordres basés sur de nombreux caractères incluant la morphologie et le comportement. L'ordre des Passeriformes contient 5712 (59%) des 9672 espèces d'oiseaux récents. La plupart des Passeriformes sont de petits oiseaux terrestres qui tendent à montrer des caractéristiques morphologiques similaires, ce qui rend difficile leur classification au niveau infraordinal. Cette étude compare certaines des différences ultrastructurales des spermatozoïdes parmi des membres sélectionnés de l'ordre Passeriformes.

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Source : MNHN, Paris

About 200 million years ago, Pangaea was breaking up to form Laurasia and Gondwanaland. The dominant terrestrial life forms were, it is believed, the dinosaurs. The radiation of the birds probably began about this time. A number of traits are used to construct the taxonomic and phylogenetic relationships between the various members of the class Aves, but it has been difficult to resolve the taxonomic relationships. According to SIBLEY & AHLQUIST [32] most passerines are small land dwelling birds that feed primarily on insects, seeds, fruit or nectar. The morphology of the syrinx is used to separate the members of the suborders Tyranni and the Passeri. As birds evolved many have undergone convergent evolution to allow distantly related groups to cope with similar environmental demands while others have undergone divergent evolution so that related groups may look very different. Homologies or similarities due to common ancestor must be recognized to demonstrate taxonomic relationships. It has been known since the works of BALLOWITZ [7] & RETZIUS [29, 30] that morphological differences are exhibited by the spermatozoa of various members of the Class Aves. Each noted especially the differences in the sperm of passerine and non-passerine sperm. More recent studies of spermiotaxonomy and spermiocladistics [19] have been useful in resolving taxonomic relationships in other groups of organisms.

Since avian sperm exhibit considerably morphological variability it will be useful to examine the ultrastructure of sperm as it relates to the phylogeny. A number of studies have given descriptions of the ultrastructure of spermatozoa of representatives of orders of non-passerine birds. The literature contains a few reports of ultrastructure of passerine spermatids and sperm in sections of testis. There is currently very little information on the ultrastructure of mature passerine sperm. The Order Passeriformes contains approximately 59% of all of the species of living birds, and the order is divided into two suborders and generally into 45 families [31, 32]. It is of interest to examine and compare the ultrastructure of the mature sperm of some representative members. Reconstructing the phylogenetic history of members of the order is a major challenge. In this paper I will follow the classification of SIBLEY *et al.* [32] in which the order Passeriformes is divided into the Suborders Tyranni (= Suboscines; Oligomyodi) and Passeri (= Oscines; Polymyodi). The suborder Passeri includes the parvorders Passerida (which includes three superfamilies and 21 families. The suborder Corvida includes three superfamilies and 15 families. MCFARLANE [23, 24] noted that the avian sperm exhibit considerable species variation in both size and morphology, thus it is here considered that the structure of sperm should yield phylogenetic information. ASA & PHILLIPS [3] described some differences between the oscines and sub-oscines.

The sperms of non-passerine birds are generally nearly linear in the head region while the sperms of passerine birds are helically coiled. Tripepi and Perotta [35] examined oscine spermiogenesis and divided the oscines (Passeri) into two types based on the presence of a granular body and the ratio of length of the nucleus to the length of the acrosome. The present work will describe the morphological variation of the sperm of various passerine birds and will compare these sperm with those of some non-passerine birds.

MATERIALS AND METHODS

Sperm specimen were obtained from various species of birds of the Order Passeriformes. All birds were from the suborder Passeri, and represented five different families. Species included *Agelaius phoeniceus, Molothrus ater, Quiscalus quiscula, Sturnus vulgaris, Cardinalis cardinalis,* and *Passer domesticus.* Male birds were obtained during the breeding season, euthanased, and sections of the distal portion of the vas deferens and/or the seminal glomeruli were removed, fixed in 2% glutaraldehyde in phosphate buffer, pH 7.2, post fixed in 1% osmium. For TEM specimen were dehydrated in acetone, embedded, sectioned, stained with uranyl acetate and lead citrate, and examined with a Philips 300. For SEM the fixed specimen were dehydrated, critical point dried sputter coated and examined with an AMRAY 1200.

Source : MNHN, Paris

RESULTS

The sperm of each species is characterized by being long and cylindrical in overall morphology, with the diameter of the acrosome, nucleus and midpiece being nearly the same. Table 1 gives the various features and measurements of the sperm.

The sperm possess a helical shape, especially in the region of the acrosome and nucleus, generally with the helical coiling ending at the neck. The degree of coiling and the length between turns of the helix is variable in sperm of different species. The sperm also possess a helical membrane starting near the anterior tip of the acrosome and extending over the length of the acrosome to the anterior end of the nucleus. The helical appearance of the midpiece and tail generally is due to the coiling of an elongated granular mass followed posteriorly by the mitochondrial sheath surrounding the neck and midpiece, and extending posteriorly to the endpiece.

The acrosome at the anterior end of the head is characterized by having an electron dense core surrounded by a more electron lucent region. Considerable differences are noted in the length of the acrosome in relation to the length of the nucleus. In some species there is an anterior nuclear fossa of variable depth to 1.1 µm. A perforatorium was not observed in any of the passerine sperm. The neck of the sperm do not appear to have typical distinct centrioles. The flagella are composed of an axoneme having a 9+2 arrangement, surrounded by 9 dense accessory fibres extending from the neck to the posterior end of the principal piece. A posterior nuclear fossa does not appear to occur in the sperm of these passerines studied. Morphology of passerine sperm differs greatly from that of the sperm of non-passerine birds.

TABLE 1. — The sperm of passerine birds exhibit considerable variation in size and morphology. Measurements from scanning and transmission electron micrographs are presented.

Family	Passeridae	Tyrannidae	Hirundinidae	Paridae	Vireonidae	Turidae	Thraupidae
Taxon	*Passer domesticus*	*Tyrannus verticalis*	*Tachycineta thalassina*	*Parus bicolor*	*Vireo olivaceus*	*Turdus migratorius*	*Piranga rubra*
Common name	House Sparrow	Western Kingbird	Violet-green Swallow	Tufted Titmouse	Red-eyed vireo	Robin	Summer Tananger
Sperm length	77 µm	55 µm	285 µm	90 µm	80 µm	70 µm	170 µm
Acrosome length	6-7 µm	2.5 µm	13.5 µm	7.1 µm	8.0 µm	6.7 µm	12 µm
Acrosomal Core	Yes		Dense core				
Nuclear Length	6 µm	14 µm	4.5 µm	5.8 µm	5.5 µm	2.5 µm	3 µm
Granular material at midpiece	yes						
Midpiece Length	55 µm	4 µm	granular material at midpiece	50 µm	10 µm	46 µm	146 µm
Helical Shape	yes	yes	yes	yes	yes	yes	yes
Helical Membrane	Yes -only on acrosome		Yes-acrosome only	acrosome only	acrosome only	acrosome only	acrosome only
Perforatorium	No		no				
Anterior Nuclear Fossa	Yes 0.7-1.08 µm		yes 0.7 µm	no	no	no	no
Posterior Nuclear Fossa	no	yes shallow	no	slight	no	no	no
Centrioles	Distal only		distal only	distal			
Accessory Fibres	yes-attached to mitochondrion	yes	yes - attached to axoneme at anterior end	yes	yes	yes	
Mitochondria	1 elongated helical - posterior to the granular material		1 or 2, long one is helical		1 helical	Helical large	
Annulus	not observed		no				
Reference	Present study	[24]	[24]	[24]	[24]	[24]	[24]

Source : MNHN, Paris

TABLE 1, continued

Family	Emberizidae	Sturnidae	Icteridae	Icteridae	Icteridae	Tyrannidae
Taxon	*Cardinalis cardinalis*	*Sturnus vulgaris*	*Molothrus ater*	*Agelaius phoeniceus*	*Quiscaous quiscula*	*Myiarchus crinitus*
Common Name	Cardinal	Starling	Brown-headed cowbird	Red-winged blackbird	common grackle	Great Crested Flycatcher
Sperm length	76 µm	50 µm	62 µm	110 µm	48 µm	50 µm
Acrosome length	6.6 µm	2.5 µm	6.0 µm	8.0 µm	7.0 µm	2.5µm
Acrosomal Core						Uniform throughout
Nuclear Length	4.0 µm	3.0 µm	3.0 µm	5.0 µm	4.0 µm	18.5 µm
Granular body at midpiece		yes	yes		yes	
Midpiece Length	35. µm		24. µm			3.3µm
Helical Shape	yes	yes	yes		yes	Acrosome, nucleus & midpiece
Helical Membrane	yes	yes	yes		yes	Acrosome only reduced size
Perforatorium						No
Anterior Nuclear Fossa			shallow		shallow	
Posterior Nuclear Fossa			yes		yes	shallow
Centrioles					distal	–
Accessory Fibers			yes		yes	Yes
Mitochondria			spiral			Helical One revolution
Annulus						none
Reference	Present study	Present study	Present study	Present study	Present study	[24]

DISCUSSION

General

The overall length of the sperm of passerine birds is highly variable, the extremes noted in those studied are 48 µm for the *Quiscalus quiscalus* sperm to 285 µm for the *Tachycineta thalassina* [24] sperm. The overall appearance ranges from having a helical structure only in the acrosomal region to having a helical acrosome, nucleus and a helical midpiece.

The acrosome of all of the passerine birds in this study is a solid, helical apical body with a helical membrane generally running from the acrosome tip to the posterior end of the acrosome. The helical membrane is a lateral extension of the acrosome and forms a left handed helix. The size of this helical membrane is highly variable. Helical membranes or helically elongate nuclei are also recorded in a variety of invertebrates such as the cephalopod and gastropod molluscs [14], chilopods [18] and leeches [12]. The overall length of the acrosome is highly variable ranging from 2.5 µm in the sperm of *Sturnus vulgarus* to a long structure that dominates the head and is several times as long as the nucleus in the sperm of *M. crintus*. The acrosome generally appears to be covered by an outer acrosomal membrane which lies just beneath the plasma membrane. The acrosomal matrix generally has two different components that can be recognized by their density in the electron beam. The acrosomes have a dense inner core and a less dense outer cortex, there is not a membrane or a space separating these regions. This is in contrast to the acrosome of the domestic fowl sperm in which there is a dense outer cortex and a less dense inner core. The less dense outer material is continuous with the space in the helical membrane. MCFARLANE [24] described membrane-limited vacuoles in the contents of the acrosome of *C. carolinus*, Vacuoles were not observed in any sperm in the present study.

Source : MNHN, Paris

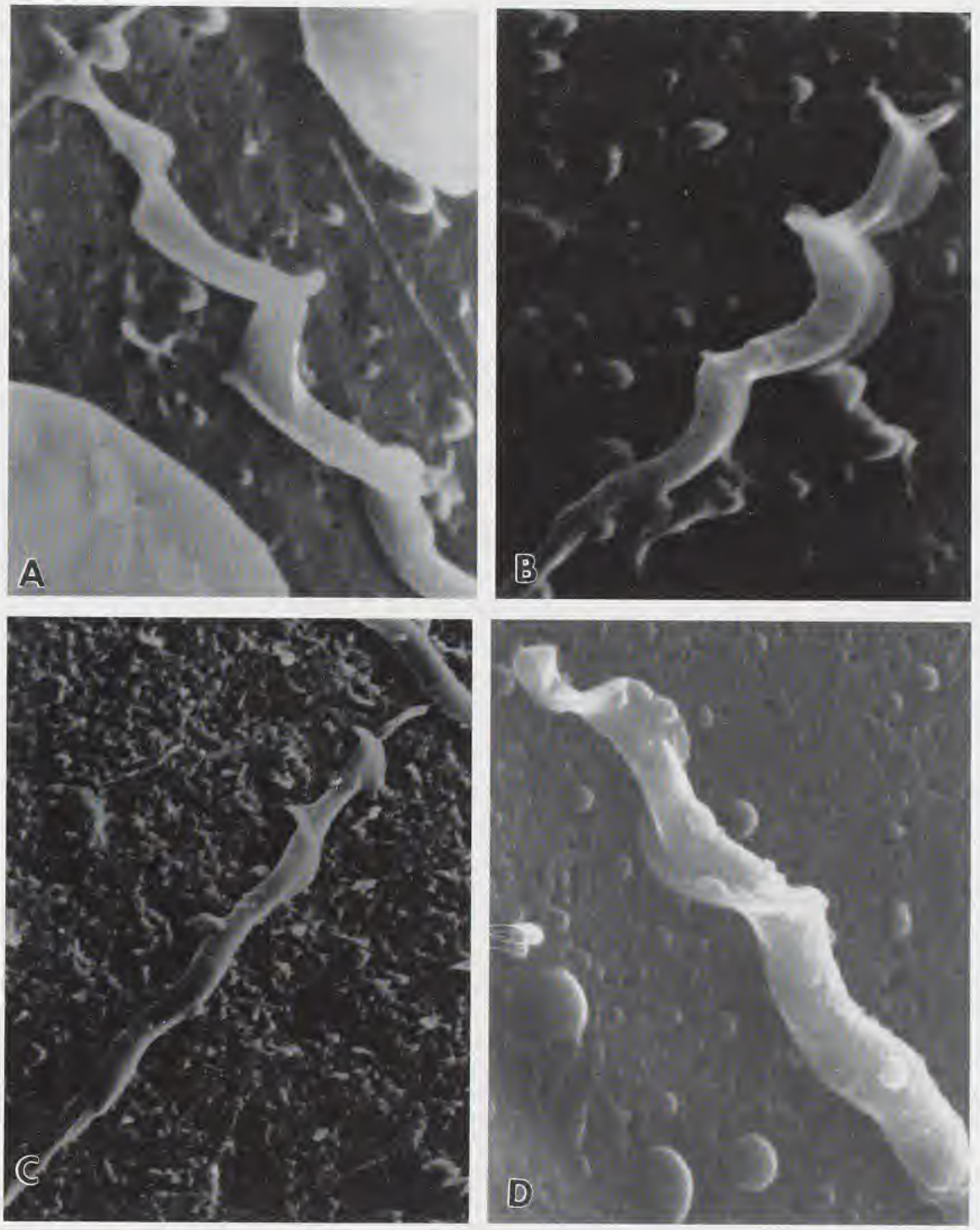

FIG. 1. — Sperm of four species of passerine birds were fixed on filters, dehydrated, critical point dried, coated with gold-palladium, and examined with a scanning electron microscope. The figure shows the acrosomal region. Note that some sperm, have a nearly linear acrosomal axis while others have a spiral shaped axis; all have a helical membrane. **a:** *Quiscalus quiscula*, Common grackle. x 8 200. **b:** *Agelaius phoeniceus*, Red-winged Blackbird. x 10 500. **c:** *Passer domesticus*, House Sparrow. x 11 200. **d:** *Cardinalis cardinalis*, cardinal x 11 200.

Source : MNHN, Paris

Nuclei

The nuclei of the sperm of passerine birds tend to be continuous with the helical shape of the acrosome and midpiece; the wavelength of the nuclear axis is highly variable and is probably characteristic of the family [22, 24]. As an example, the nucleus of the sperm of *Passer domesticus* makes one complete helical revolution over its total length. The nucleus is enclosed in a nuclear envelope which is associated closely with the plasma membrane. The chromatin condenses during spermiogenesis. In the mature sperm the nuclear material is rarely homogeneous, usually containing a number of small irregular cavities, randomly distributed, which appear to result from incomplete condensation of the chromatin.

In passerine sperm the nucleus may show a distinct helical shape. The nucleus of most non-passerine sperm is nearly straight although it may demonstrate a slight curvature, as in *Gallus domesticus*, but it is not helically coiled.

The sperm of passerine birds may have a small anterior nuclear fossa, a cone shaped depression into which the posterior extent of the acrosome extends. There are no endonuclear canals present in passerine bird sperm.

Midpiece

The centriolar region has been called the juxtanuclear apparatus by SOTELO & TRUJILLO-CENÓZ [33].

The components in the neck of the passerine sperm are not easily distinguished. SOTELO & TRUJILLO-CENÓZ [33] gave a partial description of the ultrastructure during spermiogenesis for the house sparrow. There are some modifications in the neck as the spermatids mature. Passerine sperm generally appear to lack a proximal centriole. They have a juxtanuclear body which is sometimes referred to as a modified distal centriole. This juxtanuclear body in the mature sparrow sperm appears as a striated, inverted conical structure when viewed in longitudinal sections. In cross sections the juxtanuclear body appears as irregular fibrils attached to the inner margins of the accessory fibres. The axonemal axis does not extend into the juxtanuclear body.

The axoneme is composed of the classical 9+2 arrangement of microtubules, originating a short distance posterior to the juxtanuclear body. The axoneme is surrounded by nine outer accessory fibres. The presence of these accessory fibres in passerine sperm is a feature that distinguishes them from the sperm of non-passerine birds. The accessory fibres have a tear drop shape in cross section with the point toward the associated pair of axonemal microtubules. The anterior ends of the accessory fibres form a connecting piece at the base of the nucleus, and surround the juxtanuclear body [10, 21, 24, 25]. Nine extremely short accessory fibres have been described in the sperm of certain primitive birds, located adjacent to the axoneme in the region of the annulus. These primitive bird sperm also contain a dense fibrous sheath which is located immediately posterior to the annulus and which surrounds the accessory fibres [5]. Very short accessory fibres have also been described in sperm of Galliformes [34] and the mallard duck [17]. These fibres are greatly reduced in both diameter and length when compared with the accessory fibres of passerine sperm. Outer accessory fibres are also found in the sperm of mammals [8, 26], of Tuatara [16], and in molluscan sperm [15]. In passerine birds the fibres are all of similar size and shape whereas the fibres in molluscs are of various sizes in the same sperm and in reptiles the doublets at position 3 and 8 are enlarged [16]. These outer accessory fibres in passerine sperm appear to be relatively rigid. When living passerine sperm are observed in a physiological saline or in water they appear to maintain a rigid straight morphology. The movement appears to be due to the circular motion of the rigid spermatozoon.

A spiral granular mass is present in the anterior region of the midpiece of some passerine sperm [17, 21, 24, 33]. In sperm of some species this granular mass forms a spiral mass located anterior to the nitochondrion, as observed in *Passer domesticus*, with the anterior end of the mitochondrion being a few micrometres caudal to the nucleus. In contrast, in other species the

Source : MNHN, Paris

anterior end of the mitochondrion is at the neck and the granular mass is located around the mitochondrion and the axoneme for a short distance.

All passerine sperm appear to have a single very elongated mitochondrion that may extend almost to the end of the midpiece. In the mature sparrow sperm the mitochondrion extends nearly to the end of the tail and the principal piece is nearly non-existent. The mitochondrion appears to attach to the accessory fibres associated with the axoneme at each end. The mitochondrion shows a gradual decrease in its diameter near the posterior end of the flagellum. The cristae in the mitochondrion are tubular in form and show a random arrangement when sectioned material is viewed.

An annulus was not observed in any of the passerine sperm studied. An annulus is present during spermiogenesis in the grackle [21] but it has not been observed in the mature sperm. The sperm of psittaciforms also lack an annulus [20] but they show a clearly demarcated midpiece-tail junction. In the passerine sperm the posterior end of the midpiece is demarcated by the end of the single helical mitochondrion.

If the sperm of passerine birds are compared with those of the non-passerine birds the notable differences include the presence of the helical membrane in the passerines (representatives in two orders of non-passerine birds do possess helical sperm, the Charadriiformes and Procellariformes, but it is believed that this membrane arose independently of the membrane in passerine sperm), the helical shape of the nucleus, the lack of a perforatorium , the lack of the endonuclear canals, the lack of distinct centrioles, the presence of the nine accessory fibres connected to a juxtanuclear apparatus and the lack of an annulus.

Passerine sperm differ significantly from the sperm of the non-passerines but an ultrastructural examination of spermatozoa of many more passerine species is necessary to reveal phylogenetic relationships between the passerine birds.

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Source : MNHN, Paris

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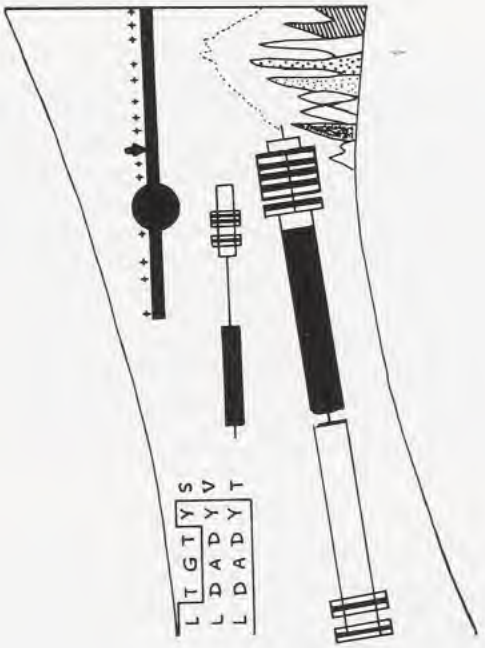
Source : MNHN, Paris

Proteins



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Histone H1 and the Evolution of the Nuclear Sperm-Specific Proteins

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ABSTRACT

The chromosomal proteins of the sperm exhibit an enormous structural variability. During the last ten years, important progress has been made in the chemical and physical characterization of these proteins over a wide range of organisms. The data emerging from these studies indicate that the nuclear sperm-specific proteins are not as heterogeneous as originally thought. Indeed, from the compositional point of view they can be arranged in a discrete number of basic types: H Type (histone), P Type (protamine) and PL Type (protamine-like) consisting of proteins with an intermediate composition between histones and protamines. Research on the PL Type has been carried out mainly in molluscs. This taxonomic group is of special interest because it contains organisms with nuclear sperm-specific proteins that can be considered representative of each of the three protein types. Work carried out with bivalve molluscs suggests the possibility that all members of the PL Type have evolved or are related in one way or another to a PL-I protein with structural characteristics of an H1 histone. PL-I proteins have been recently identified in both protostomes and deuterostomes. The structural links existing amongst PL proteins, their arginine-rich composition and their relationship to chromosomal proteins of the histone H1 family suggest an evolutionary relationship amongst the three basic protein types. On this basis, an evolutionary pathway starting from an early histone precursor and leading to the protamine type which is present in the most evolved organisms of the deuterostome and protostome branches is proposed.

RÉSUMÉ

Histone H1 et évolution des protéines nucléaires spécifique des spermatozoïdes

Les protéines chromosomiques des spermatozoïdes montrent une immense variabilité structurale. Pendant les dix dernières années, d'importants progrès ont été faits dans la caractérisation chimique et physique de ces protéines dans des organismes très variés. Les résultats émergeant de ces études montrent que les protéines spécifiques des spermatozoïdes ne sont pas aussi hétérogènes que ce que l'on pensait originellement. En fait, elles peuvent être classées par leur composition en un nombre fini de types: type H (histone), type P (protamine) et type PL (proche des protamines) correspondant à des protéines ayant une composition intermédiaire entre les histones et les protamines. La recherche sur les protéines proches des protamines a porté principalement sur les Mollusques. Ce groupe taxonomique est d'un intérêt particulier car il comprend des organismes avec des protéines spécifiques des spermatozoïdes qui peuvent être considérées comme représentant chacun des trois types. Les travaux sur les Mollusques Bivalves suggèrent la possibilité que toutes les protéines du type PL ont évolué ou sont reliées d'une manière ou d'une autre avec une protéine PL-I avec les caractéristiques structurales d'une histone H1. Les protéines PL-I ont été récemment identifiées chez les Protostomiens et les Deutérostomiens. Les liens structuraux existant entre les protéines PL, leur composition riche en arginine et leurs relations avec les protéines chromosomiques de la famille des histones H1 suggèrent une relation évolutive entre les trois types de protéines. Sur cette base, un chemin évolutif commençant avec un précurseur histone et menant au type protamine qui est présent chez les organismes les plus évolués des Deutérostomiens et des Protostomiens est proposé.

AUSIO, J., 1995. — Histone H1 and the evolution of the nuclear sperm-specific proteins. *In*: JAMIESON, B. G. M., AUSIO, J., & JUSTINE, J.-L. (eds), Advances in Spermatozoal Phylogeny and Taxonomy. *Mém. Mus. natn. Hist. nat.*, **166** : 447-462. Paris ISBN : 2-85653-225-X.

Source : MNHN, Paris

During spermatogenesis, chromatin from the stem cells undergoes several dynamic transitions which are often associated with important changes in its composition and structure. In most instances, the composition of the chromosomal proteins at the onset and in the final stages of spermatogenesis is quite different. These compositional changes significantly alter the structure of chromatin. As a result, chromatin becomes highly compacted and gene expression is completely shut off in the spermatozoon. The ways in which all this is achieved can be mediated by a wide spectrum of apparently diverse chromosomal proteins [17, 40] which are mirrored by the morphological diversity of the mature sperm cell [15]. The structure of chromatin arising from the protein-DNA interactions in each particular situation is in most instances poorly understood and the evolutionary relationship amongst these proteins still remains obscure.

This chapter reviews the classification of the nuclear sperm-specific proteins in the light of new biochemical data that have been gathered in recent years. With the information on the chromosomal sperm-proteins expanding over a wider range of taxonomic groups from both the protostome and deuterostome branches as well as in lower phylogenetic taxa, it is also possible to envisage and outline an evolutionary relationship for these proteins.

RESULTS AND DISCUSSION

Structural variability and compositional homogeneity of the nuclear sperm-specific proteins. The classification of the nuclear sperm-specific proteins, 25 years after.

In 1969, David BLOCH [17] published the first comprehensive catalogue of the nuclear sperm-specific proteins. Despite the attempt at classification proposed in his paper (Fig. 1), the author concluded: "...the variability (non-conservatism) of the protein reflects an evolutionary indifference to a relatively unimportant protein in an inert nucleus". This somehow pessimistic comment reflects BLOCH's difficulty in finding a phylogenetic relationship among the different groups of chromosomal sperm proteins. This was mainly due to the fact that even though proteins belonging to the same group in the classification had a similar amino acid composition, they exhibited an enormous structural variability. In addition, the patchy cytochemical and biochemical information, available at that time, came from organisms usually belonging to phylogenetically distant groups.

The availability of techniques such as high performance liquid chromatography and protein microanalysis has allowed us to extend this information to a much broader spectrum of organisms in recent years. More importantly, it has been possible, for the first time, to gain information on organisms from phylogenetically relevant/related taxonomic groups [59, 60].

The global picture emerging from these studies is that despite their enormous structural variability, the nuclear sperm-specific proteins can be grouped, from the compositional point of view, into three basic categories (Fig. 1). Type H (Histone type) consists of proteins with amino acid compositions that, although specific for the germ line, are structurally and compositionally similar to those of the somatic histone type (Table 1). This grouping is equivalent to the *Rana* type of BLOCH's classification [17] (see also Fig. 1). At the other end of this classification, type P (protamine type) consists of proteins of low molecular mass (4000 to 10 000 daltons), that are arginine rich (arginine content $\geq 60\%$). The P type is defined here irrespective of the presence or absence of cysteine in the protein molecule. Thus it brings together under a common name the monoprotamine and stable protamine type of BLOCH's classification [17] (Fig. 1). In the course of spermatogenesis, these proteins almost completely replace the histone counterpart of the stem cells (see [53] for a recent review on these proteins, and Table 3). The PL type (protamine-like) consists of proteins with an amino acid composition intermediate between the previous types. The arginine and lysine content usually amounts to at least 35-50% of their amino acid composition, but occasionally it can be higher. Although the relative ratio of these two amino acids may vary over a wide range, it usually stays constant for a given taxonomic group (Table 2). In

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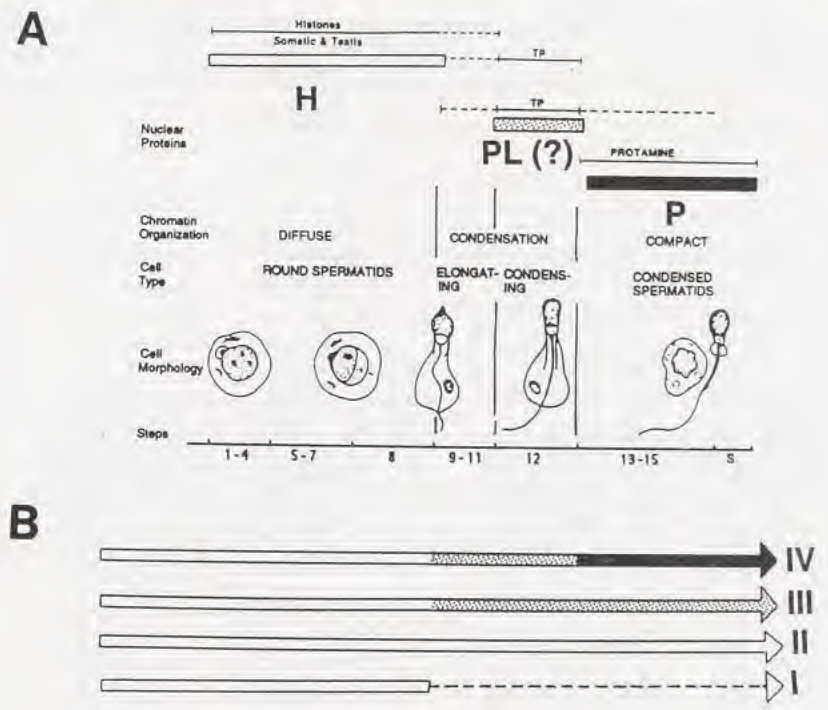

C

BASIC TYPES OF NUCLEAR SPERM-PROTEINS

	0. Relatively non-basic proteins (Crab type)
1. H type (Histones)	1. Typical histones (Rana type)
2. PL type (protamine/histone like)	2. Intermediate proteins (Mytilus type)
3. P type (protamines)	3. Monoprotamines (Salmon type)
	4. Stable protamines (Mouse type)

FIG. 1. — **A:** Schematic representation of the changes in the nucleus in ram spermiogenesis (modified from [42]). TP = transition proteins. **B:** Nuclear protein transitions in the four basic types of spermiogenesis. **C:** Classification of the nuclear sperm-proteins. The classification used in this paper is compared to that proposed by BLOCH [17].

most instances, these proteins can be structurally related to histone H1 (as will be discussed later) and as in the case of the P type these proteins replace to a large extent (≥80%) the somatic histones during spermiogenesis. In the final stages of spermatogenesis, PL proteins exhibit a significant degree of structural heterogeneity, with molecular masses in the 5 000-30 000 dalton range. This group corresponds to the type of intermediate proteins (*Mytilus* type) of BLOCH's classification [17] (see also Fig. 1).

The phylogenetic relevance of this later group of proteins was already anticipated by BLOCH [18]. He noted "... the similarities between the 'evolutionary intermediate' proteins of the mature sperm of mussel and the 'developmentally intermediate' proteins of the spermatids of *Loligo*

Source : MNHN, Paris

TABLE 1. — Amino acid composition (mol %) of the H-type proteins from the sperm of several representative organisms in comparison with somatic type H proteins from calf thymus.
LP = *Limulus polyphemus* [48], PM = *Petromyzon marinus* [60], CA = *Carassius auratus* [49] and CT = calf thymus [45].
tr = trace amounts.

	H1						H2A				H2B				H3			H4			
	LPa &b	LPa	LPc	PM	CA	CT	LP	PM	CA	CT	LP	PM	CA	CT	PM	CA	CT	LP	PM	CA	CT
Lys	28.7	24.1	20.0	27.6	29.2	**26.8**	10.2	9.7	10.5	**10.2**	14.2	14.1	14.0	**14.1**	8.3	10.0	**10.0**	10.1	10.4	10.1	**11.4**
His	-	-	-	-	0.5	-	1.8	1.6	2.0	**3.1**	2.6	3.3	2.0	**2.3**	1.7	1.4	**1.7**	2.3	1.8	2.0	**2.2**
Arg	3.2	2.7	6.7	2.2	2.1	**1.8**	8.6	9.8	8.6	**9.4**	6.8	8.2	7.8	**6.9**	13.8	13.2	**13.0**	12.1	13.4	12.5	**12.8**
Asx	2.5	3.5	6.1	3.9	2.6	**2.5**	6.6	6.3	6.1	**6.2**	5.8	4.8	5.2	**5.0**	4.2	4.4	**4.2**	5.6	4.8	5.3	**5.2**
Thr	6.4	7.4	6.0	3.6	4.0	**5.6**	2.6	3.2	5.2	**3.9**	6.9	6.6	6.2	**6.4**	7.5	6.4	**6.8**	6.3	6.7	6.5	**6.3**
Ser	6.5	8.8	9.4	7.3	6.0	**5.6**	4.3	5.2	5.0	**3.4**	12.5	10.3	7.0	**10.4**	4.5	5.1	**3.6**	4.6	2.0	2.7	**2.2**
Glx	5.3	7.9	10.1	4.7	4.0	**3.7**	10.8	9.4	9.5	**9.8**	9.2	6.4	8.6	**8.7**	12.0	11.7	**11.6**	8.1	6.5	6.8	**6.9**
Pro	11.2	8.8	8.7	9.7	9.3	**9.2**	4.9	4.1	5.7	**4.1**	2.2	6.2	4.8	**4.9**	4.5	5.0	**4.6**	1.6	1.4	1.6	**1.5**
Gly	5.5	6.8	5.8	4.0	5.4	**7.2**	11.2	11.4	10.2	**10.8**	5.1	5.9	7.9	**5.4**	5.6	6.3	**5.4**	16.4	16.8	14.7	**14.9**
Ala	15.4	15.8	10.0	21.8	22.6	**24.3**	12.7	13.1	13.7	**12.9**	12.3	11.6	11.6	**10.8**	13.3	13.0	**13.3**	7.4	7.9	8.0	**7.7**
1/2 Cys	-	-	-	-	-	-	tr.	-	-	-	-	-	-	-	0.6	-	**1.0**	tr.	-	-	-
Val	5.8	5.6	4.6	6.8	4.4	**5.4**	6.6	7.2	6.2	**6.3**	6.3	6.0	6.7	**7.5**	4.2	4.6	**4.4**	7.0	8.1	8.4	**8.2**
Met	-	-	1.2	-	-	-	-	0.5	-	-	1.4	2.1	-	**1.5**	0.8	-	**1.1**	tr.	0.2	-	**1.0**
Ile	2.8	3.1	3.8	1.1	2.0	**1.5**	4.5	3.7	3.3	**3.9**	4.6	5.8	4.7	**5.1**	4.7	5.0	**5.3**	5.1	5.5	5.4	**5.7**
Leu	3.0	3.3	5.2	5.3	4.7	**4.5**	12.1	11.9	11.4	**12.4**	4.6	4.5	6.5	**4.9**	8.9	8.8	**9.1**	7.4	8.6	8.5	**8.2**
Tyr	2.5	1.0	1.5	0.8	0.9	**0.9**	2.0	2.3	1.8	**2.2**	2.8	2.8	3.8	**4.0**	2.2	2.0	**2.2**	2.9	3.6	3.6	**3.8**
Phe	1.2	1.0	1.3	1.1	0.8	**0.9**	1.0	0.9	0.7	**0.9**	2.5	1.6	1.6	**1.6**	3.3	2.7	**3.1**	2.0	2.1	2.1	**2.1**
Trp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

(squid) [16] and salmon"... (which both contain proteins of the P type in their mature sperm)... "suggest a relationship between ontogeny and phylogeny". Although the precise relationship (if any) between the "developmentally intermediate" proteins and PL proteins has yet to be established, BLOCH's hypothesis provides an excellent model for the biochemical classification of spermatogenesis within a phylogenetic context (Fig. 1B). Thus, from the point of view of the nuclear sperm-specific proteins which are present in the mature sperm and the protein transitions undergone by chromatin during the differentiation process, four basic types can be defined. The first group (type I) of this classification consists of those organisms which lose their chromosomal protein complement (histones) during spermiogenesis. As a result, DNA appears essentially naked in the mature sperm cell, which exhibits an uncondensed nucleus. In Type II, the somatic type (H type) of histones is maintained throughout the whole spermatogenesis. In most instances histones undergo an important turnover during which the somatic histones from the stem cells at the onset of spermatogenesis are partially or completely replaced by sperm-specific histones (Table 1). In the third group of this classification, the somatic and/or the germinal histone type of proteins (H type), is replaced to a large extent by proteins of the PL type. These are the proteins that are found in the mature spermatozoa. Finally, in group IV, at the onset of spermiogenesis, the histones of somatic and/or germinal type are initially displaced by a set of intermediate proteins "transition proteins" and finally replaced by arginine rich proteins of the protamine type.

With the exception of group I, which has been described so far only in some members of the decapod crustaceans [72], all the other types of this classification are widespread in both deuterostomes and protostomes.

Source : MNHN, Paris

TABLE 2. — Amino acid composition (mol %) of some representative proteins of the PL-type. tr = trace amounts.

Group	Molluscs			Tunicate	Cephalochordate
Species	*Ensis minor*	*Mytilus californianus*	*Macoma nasuta*	*Styela plicata*	*Branchiostoma floridae*
Reference	[33]	[47]	[3]	[59]	[60]
	PL-I (EM-6)	PL-III	PL-III	P1	
Lys	24.9	20.5	25.2	14.3	24.7
His	4.0	-	0.2	1.1	-
Arg	21.7	32.1	27.9	32.5	25.3
Asx	2.4	-	3.5	4.6	-
Thr	2.0	2.5	-	1.2	-
Ser	18.9	15.4	28.7	4.6	16.5
Glx	1.8	-	0.9	2.2	-
Pro	tr	5.1	0.6	1.4	5.6
Gly	2.6	7.5	1.8	13.2	6.1
Ala	12.7	15.9	9.5	7.1	21.7
1/2 Cys	-	-	-	0.7	-
Val	2.3	1.1	0.4	4.6	-
Met	0.9	-	0.1	1.4	-
Ile	1.2	-	0.5	2.4	-
Leu	2.9	-	0.5	4.5	-
Tyr	0.6	-	0.1	2.2	-
Phe	1.0	-	0.2	2.2	-
Trp	-	-	-	-	-

Presence of an H1-like PL-protein in the sperm of Molluscs

The study of the nuclear sperm-specific proteins from molluscs is of special interest for several reasons. First, this taxonomic group consists of organisms that can be considered representative of any of the protein types described in the previous section. Also, this is the group from which the organism *Mytilus* (mussel) was selected by BLOCH to name the protein type corresponding to PL-type of the protein classification described in the preceding section (Fig. 1).

With regard to the first point, all the organisms of the subclass Pteriomorphia (oysters and scallops) of the class Bivalvia analyzed to date belong to the H type [2, 4, 52, 76]. All the members of the class Cephalopoda seem to contain chromosomal proteins of the P-type (Table 3), some of which may even contain cysteine, as in the case of *Eledone* [68]. Cysteine is also present in the mammalian P-type proteins. However, the most common proteins present in the sperm of the organisms from this phylum are by far the proteins from the PL-type. From a pragmatic point of view, these latter proteins can be classified into two major categories: those PLs which have a molecular mass smaller than 15,000 daltons and those with higher molecular mass. Although this division may seem arbitrary, it has profound structural implications, as will be described next.

Source : MNHN, Paris

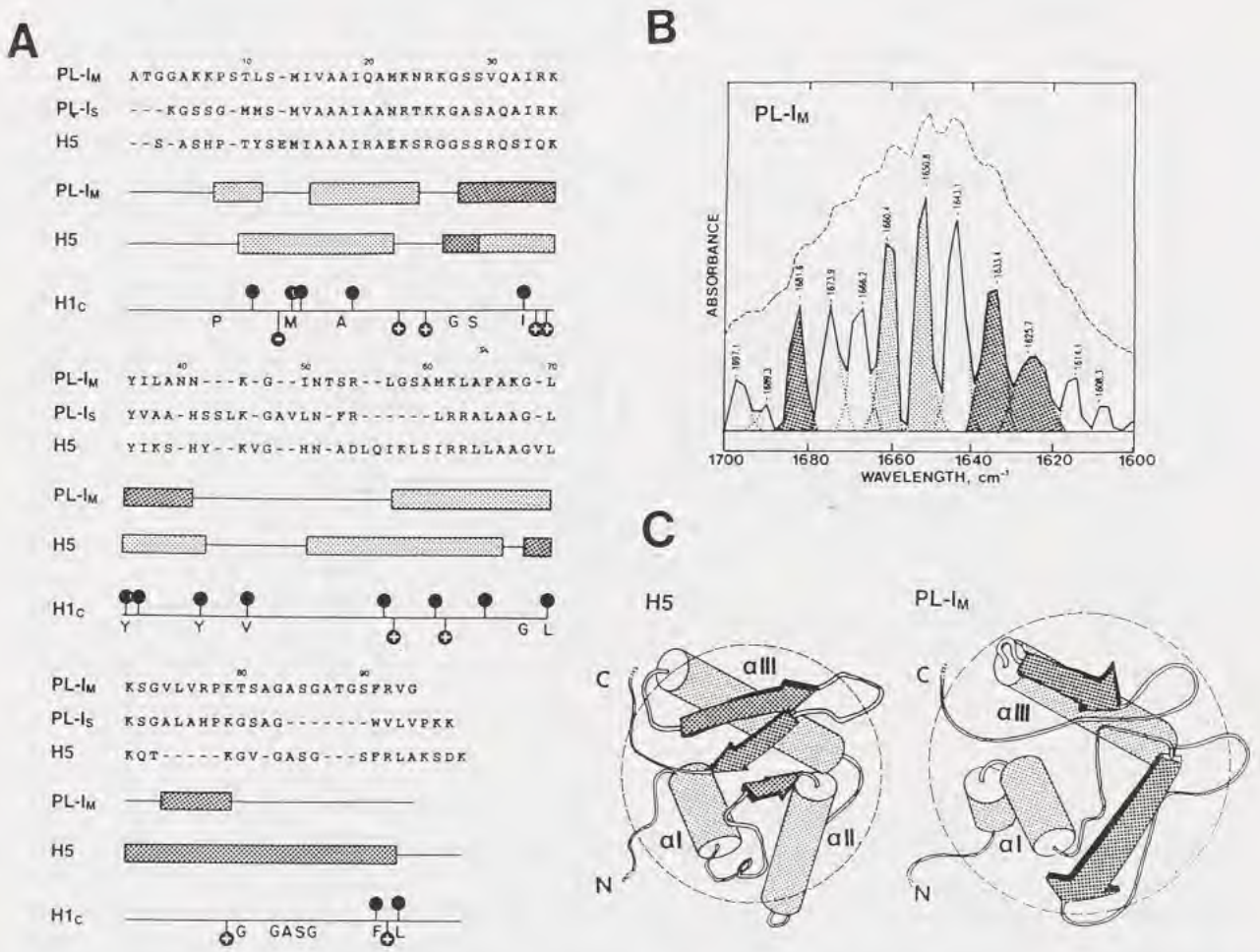

FIG. 2.— **A:** Sequence comparison between the trypsin-resistant cores of two PL-I proteins from bivalve molluscs (PL-I_M from *Mytilus californianus*, PL-I_S from *Spisula solidissima*) and the core histone H5 from chicken erythrocyte. The shaded boxes shown below identify the α-helix (light) and ß-sheet domains as determined by combination of secondary structure prediction and the experimental analysis shown in B [39]. Also shown are the major conserved features for the core of the histones of the H1 family [27]. In this representation, the black dots correspond to conserved hydrophobic residues. Conserved positively and negatively charged amino acids are also identified. **B:** Fourier-transform Infrared Spectroscopy (FTIR) analysis of the trypsin-resistant core of PL-I from *M. californianus*. The deconvolution and curve-fitting of the region corresponding to the amide I band of the infrared spectrum is shown. The different frequencies (peaks) of the spectrum can be assigned to different secondary structures [39]. α-helix (lightly shaded), ß-sheet (darkly shaded), other (not shaded). The areas under the different frequencies (peaks) are proportional to the amount of the corresponding secondary structure. C. Tertiary structure organization of the trypsin-resistant globular core of histone H5 determined by X-ray crystallography [55]. By comparison, an idealized hypothetical model for the trypsin-resistant core of PL-I from *M. californianus* is also shown. The model is based on the conservation of the primary structure and on the secondary structure assignments shown in A.

In order to account for the large molecular mass of some of the PL-proteins found in molluscs such as *Spisula solidissima* (surf clam) (which has a PL protein with Mr ≈ 27 000), SUBIRANA *et al.* [68] had earlier proposed a mechanism of "gene polymerization" from a PL precursor gene of lower molecular mass. In 1986 we decided to test this hypothesis [12] by

Source : MNHN, Paris

TABLE 3. — Amino acid composition (mol %) of several representative proteins of the protamine type P.
* average composition from the P1 and P2 components from [70]
tr = trace amounts.

Group	Molluscs	Molluscs	Fish	Amphibians	Reptiles	Birds	Mammals
Species	*Loligo pealeii*	*Gibbula divaricata*	*Oncorhyncus keta*	*Bufo japonicus*	*Chrysemys picta*	*Gallus domesticus*	*Bos taurus*
Reference	[9]	[68]	[1]	[70] *	[23]	[50]	[24]
			Salmine				P1
Lys	-	5.8	-	5.4	1.0	-	-
His	-	-	-	9.6	4.0	-	2.1
Arg	78.0	56.3	67.2	42.1	65.3	58.5	50.0
Asx	-	-	-	2.0	-	-	-
Thr	-	2.0	-	6.9	2.5	1.6	6.3
Ser	12.2	17.1	9.9	5.7	7.0	17.2	4.2
Glx	-	-	-	4.7	4.4	-	2.1
Pro	2.4	-	9.1	9.7	tr	3.5	-
Gly	-	4.9	6.5	0.8	13.2	8.6	4.2
Ala	-	9.6	1.3	4.9	1.3	3.2	2.1
1/2 Cys	-	-	-	0.3	-	-	12.5
Val	-	3.5	4.7	5.3	-	1.7	6.3
Met	-	-	-	-	-	-	-
Ile	-	-	1.3	-	-	-	2.1
Leu	-	-	-	-	-	-	2.1
Tyr	7.3	-	-	2.7	-	6.2	4.2
Phe	-	-	-	-	-	-	2.1
Trp	-	-	-	-	-	-	-

conducting a detailed structural and biochemical analysis of the PL protein of *Spisula*. The results surprisingly showed that, like somatic histone H1, the major PL protein component from *Spisula* sperm: 1) was *not insoluble* in dilute PCA (5%) [4], 2) could be digested with trypsin to produce a 75 amino acid trypsin-resistant core, 3) this trypsin-resistant core had a globular conformation, 4) the primary structure of this globular core exhibited a high degree of sequence similarity with the globular domain of somatic histone H1 and it fulfilled the constraints imposed by the consensus sequence established for the globular trypsin-resistant domain of this histone [27] (Fig. 2). When this was taken together with the fact that the trypsin-resistant core of histone H1 was the most conserved region of this molecule [26], it was clear that, despite the high lysine and arginine content of *Spisula* PL (Table 4), this molecule was a member of the histone H1 family.

Extension of these analyses to PL proteins from other bivalve molluscs [20, 33, 34, 39] indicates that the extent of sequence similarity of the globular domain of these molecules and that of other histone H1 members is in the 30-40% range, with higher values in the case of the sperm-specific histone H1 proteins. This value should be considered high, taking into account the high evolutionary variability of the histones of the H1 family [37]. We call these proteins PL-I proteins. We define them as PL proteins (Fig. 1C) which have an internal trypsin-resistant

Source : MNHN, Paris

TABLE 4. — Amino acid composition (mol %) of the PL-I proteins of different organisms. tr = trace amounts.

Organism	*Metridium senile*	*Spisula solidissima*	*Chelysoma productum*	*Mullus barbatus*	Calf thymus histone H1	Chicken erythrocyte histone H5
Reference	Unpublished	[8]	Unpublished	[58]	[45]	[45]
Lys	11.0	24.8	17.3	24.2	26.8	23.6
His	0.5	-	1.8	-	-	1.9
Arg	25.4	23.1	20.8	22.1	1.8	12.4
Asx	2.8	0.6	5.0	5.1	2.5	1.7
Thr	4.3	4.3	2.1	4.5	5.6	3.2
Ser	11.2	21.7	6.8	9.2	5.6	11.9
Glx	5.4	0.6	4.0	tr	3.7	4.3
Pro	2.5	2.4	1.9	6.8	9.2	4.7
Gly	8.6	3.0	18.7	5.4	7.2	5.3
Ala	11.6	14.2	6.7	11.9	24.3	16.3
1/2 Cys	-	-	-	-	-	-
Val	7.6	2.3	2.9	4.9	5.4	4.2
Met	-	0.4	0.5	tr	-	0.4
Ile	2.3	0.5	3.4	tr	1.5	3.2
Leu	3.6	1.7	5.8	5.8	4.5	4.7
Tyr	1.4	0.3	1.6	tr	0.9	1.2
Phe	1.8	0.3	1.2	tr	0.9	0.6
Trp	-	0.3	-	-	-	-

globular core with structural and compositional similarity to the globular counterpart of the protein members of the histone H1 family. Unlike the sperm-specific H1 histones found in echinoderms or in the bivalve group Pteriomorphia [2, 54] (and like protamines), PL-I proteins replace most of the core and linker histones present at the onset of spermiogenesis. They account for ≥ 80% of the chromosomal protein of the mature spermatozoon.

The N and C terminal non-globular domains of these molecules are extremely variable. Most of the basic residues are found in these regions and in many instances the arginine residues are arranged in clusters similar to those found in the proteins of the protamine type [4].

All PL proteins with $M_r \geq 15{,}000$ analyzed to date are members of the PL-I protein group. When similar structural analyses as those carried out with these molecules were extended to other PL proteins with $M_r < 15{,}000$ [3], no trypsin-resistant peptide could ever be detected in these molecules, regardless of their overall compositional similarity to PL-I. This could already be anticipated from the complete absence of hydrophobic amino acids (Phe, Tyr, Leu...), which are usually present in low amounts in PL-I proteins.

Chromatin Structures

During the last twenty years important progress has been made in our knowledge of the chromatin organization of the nucleus of somatic cells [71]. In contrast, our knowledge of chromatin organization in the sperm cell has progressed at a slower pace but has benefited greatly from the information gained in the former as will be discussed later.

Source : MNHN, Paris

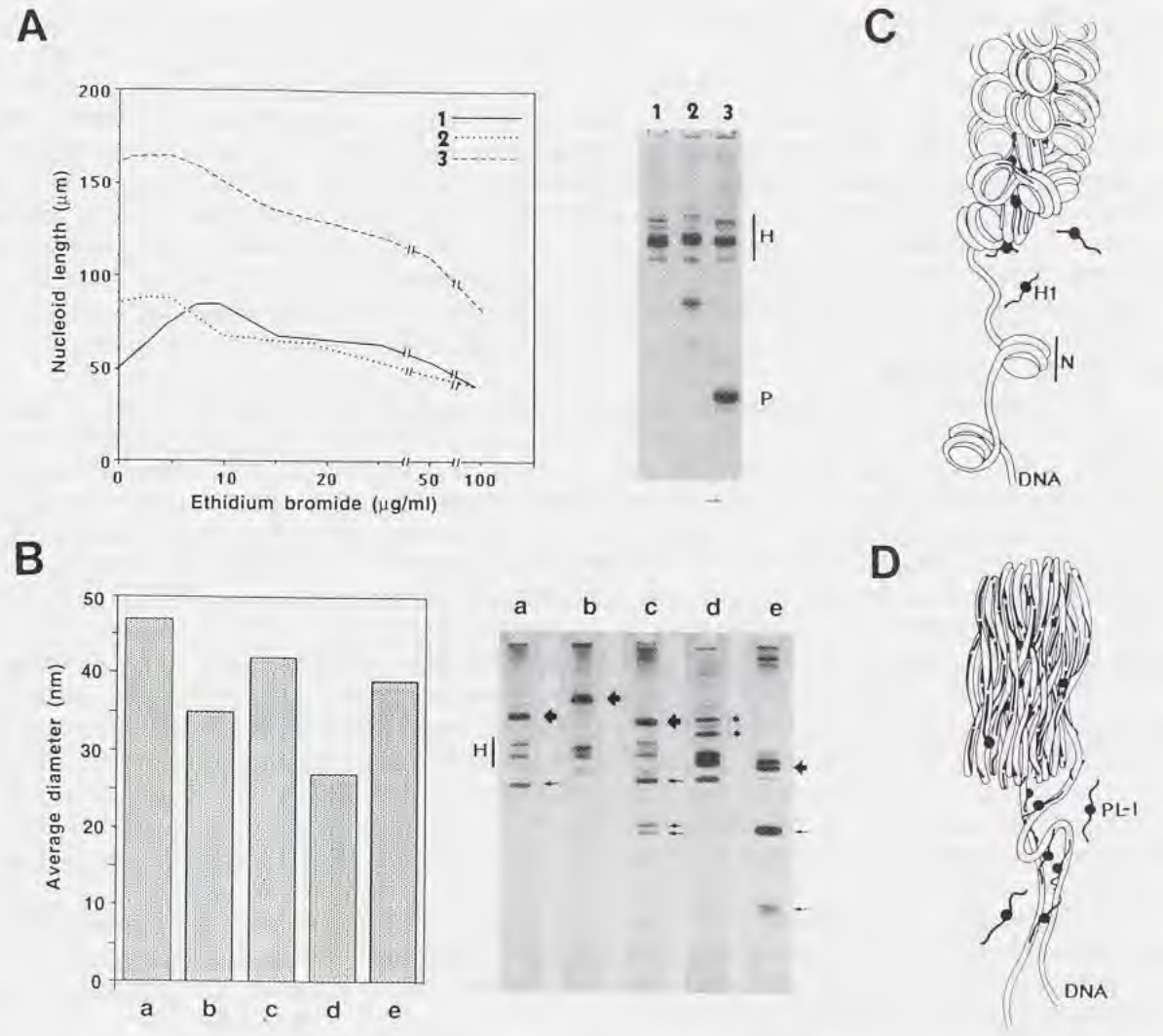

FIG. 3. — **A:** Change in the length of sperm nucleoids as a function of the ethidium bromide concentration. Figure redrawn from [56]. 1. Sperm nucleoids consisting of protein of the H type (*Rana catesbeiana*) exhibit a bipharic change. As a result of the interaction of the H type proteins (histones) with DNA, chromatin adopts a nucleosomal organization (see C). Nucleosomes stabilize DNA in a negatively supercoiled configuration. As the ethidium bromide concentration increases, the native negative superhelicity is increasingly lost (ethidium bromide intercalation twists DNA in a positive sense), DNA becomes more relaxed and the size of the nucleoid, therefore, keeps increasing until all negative supercoils have been removed (equivalence point ~8 μg/ml ethidium bromide). Further increase in the ethidium bromide beyond this point induces positive supercoiling. 2, 3. In contrast, sperm nucleoids consisting of protein of the PL (*Xenopus laevis*), 2, and P type (*Bufo fowleri*), 3, exhibit only a decrease in their dimensions as the concentration of ethidium bromide increases. This indicates that DNA has a relaxed conformation in the nucleoprotein structure arising from its interaction with these kinds of proteins. The 5% acetic acid, 2.5 M urea polyacrylamide gel electrophoresis (PAGE) shows the protein composition of the sperm nuclei of 1) *Rana catesbeiana*, 2) *Xenopus laevis*, 3) *Bufo* sp. **B:** Average diameter of the chromatin fibres from advanced spermatids of marine bivalve molluscs with different H- and PL-type protein compositions (modified from [22]). A 5% acetic acid, 2.5 M urea PAGE is also shown. a) *Ensis ensis*, b) *Callista chione*, c) *Donax trunculus*, d) *Pecten maximus*, and e) *Mytilus edulis*. The thick arrows point to the PL-I proteins and the thin arrows point to other PL protein components of smaller molecular mass. **C:** Upon interaction with histone H1, the polynucleosome filament folds into a higher order structure. This results in the 30 nm fibres observed in the sperm of organisms containing H-type chromosomal proteins. **D:** Hypothetical arrangement of the PL-DNA nucleofilaments in the (25-50 nm) chromatin fibres observed in advanced spermatids (as in B) of the organisms with a nuclear PL-type protein composition. H1, H2A, H2B, H3, H4, histone types, N, nucleosome, P, protamine.

Source : MNHN, Paris

Chromatin organization (somatic, H-type). Chromatin resulting from the interaction of DNA with proteins of the histone type (H-type) is organized in discrete nucleosome subunits. In the nucleosome, about 200 base pairs of DNA are wrapped in approximately two negative superhelical turns about a histone core octamer (consisting of "core histones" H2A, H2B, H3 and H4). Thus, DNA is stabilized by nucleosomes in a negative supercoiled state, which poises eukaryotic chromatin for genetic activity (replication, transcription). In addition to the "core histones", "linker histones" (proteins of the H1 histone family) bind to the linker DNA regions connecting the neighbouring nucleosome subunits and condense chromatin into higher order structure fibres of about 300 Å in diameter (Fig. 3C). Although electrostatic interactions play an important role in the maintenance of this organization, only about half of the negatively charged DNA phosphates are neutralized by the arginine/lysine side chains. This makes the nucleohistone complex very sensitive to environmental ionic conditions [65] and amenable for interaction with other regulatory proteins.

The realization that histones are not only passive structural blocks, but also functional elements [35] represents one of the most important landmarks in our understanding of the function-structure relationships of somatic chromatin. Despite the lack of DNA sequence specificity of the histone-DNA interactions, the resulting nucleosome structures may play, by themselves or in conjunction with other regulatory proteins [43, 75], a very important role in the modulation of the genetic activity of the chromatin complex [6]. The ability of different histone H1 (linker histones) subtypes to condense the chromatin fibre to different extents, and thereby possibly to be involved in its genetic activity, has also been postulated [25]. As will be discussed at the end of this chromatin section, the latter consideration is important in understanding the presence of proteins of the H type in the sperm of some organisms. All these functional and structural aspects of the H-type chromatin organization may explain why somatic histones have been conserved so invariably through evolution, in contrast with the proteins of the sperm.

Chromatin organization resulting from the interactions between the PL-P type proteins with DNA. I am next going to highlight briefly what I consider to be the most recent significant advances in chromatin organization resulting from the interaction of DNA with proteins of the PL and P type.

Although PL and P proteins usually coexist with a small amount of histones in the sperm nucleus [2, 14, 32], the structure of the nucleoprotein complexes arising from the interaction of these proteins with DNA lacks the nucleosomal organization of the somatic chromatin type, as can be envisaged by X-ray diffraction [7, 10, 63].

The overall negative superhelicity of DNA is lost [56] (Fig. 3A), most likely as a result of the topoisomerase II activity associated with the histone displacement/replacement by these PL or P proteins [46, 57]. Thus, the nucleohistone-nucleoprotamine (protamine-like) transition leads to a complete reorganization of chromatin, while possibly maintaining the specific three dimensional organization of DNA and its DNA loop domain structure [73].

The detailed molecular structures of the nucleoprotein (P, PL) complexes are still controversial. Both PL and P proteins interact electrostatically with DNA (which basically retains a B conformation [63]) to form fully saturated complexes, unlike the somatic nucleohistone [1, 10, 13]. In these complexes, the PL and P proteins have been postulated to adopt an α-helical like configuration [51, 66, 74]. The positioning of these proteins in the major or minor groove of DNA has not yet been experimentally settled [65]. Nevertheless, recent raman spectroscopy analysis suggest that fish protamine may adopt an unusual $1\rightarrow3$ γ turn (non α helical) structure interacting with the major groove of the DNA [36].

At the higher order level of organization, it has been shown recently [22] that PL proteins (Fig. 3B), like H type proteins, can organize the nucleoprotein complexes into 250-500 Å fibres regardless of the particular PL composition and the absence of nucleosome-like structures. This is an important finding because it indicates that the higher order structures of the nucleoprotein complexes are mainly determined by the ionic nature of the interactions involved [31, 67], rather

Source : MNHN, Paris

than the particular structure of the proteins (H, PL or P) itself. Intermediate 300-400 Å fibres have also been described during the process of chromatin condensation in the sperm of cephalopods [44], salmon [77], lizards [19] and humans [62], all containing P type proteins.

Sperm chromatin variability

After analyzing the different types of chromatin structures, the next question that arises is, what is the reason for so much chromatin variability in the sperm? Although there is no clear cut answer to this question, in what follows I will discuss several points which may provide a useful hint into the problem.

The only functional theme that all the sperm chromatin types I through IV, in Fig. 1B, have in common is the complete shut off of the genetic activity in the mature spermatozoon.

Since H type is the protein type which is always present at the onset of spermiogenesis in any of these groups, regardless of the final nuclear protein composition, it is clear from what has been mentioned earlier, that the silencing of the genetic activity at this point can be achieved in several different ways.

One possible way (type I of spermiogenesis) would be the complete erasing of the starting chromatin structure (including nucleosomes, and other chromatin associated regulatory proteins). An alternative way could be by increasing the ratio between linker histones and core histones or by using specific linker histones [54] that lock chromatin in a functionally inert structure (type II, Fig. 1B). A third possibility would be the replacement of the H type proteins by highly charged sperm-specific PL or P proteins (types III and IV of Fig. 1B) which remove the nucleosomal organization and negative superhelicity of DNA. Thus, in addition to the genetic repressive effect, types I, III and IV also have in common the erasing of the nucleosome imprinting of the stem cells. It is not clear yet whether or not in the case of type II a reorganization (randomization) of the nucleosome positioning takes place upon binding of the specific linker histones, which could produce a similar erasing effect.

While we do not know exactly why type P has been increasingly selected throughout evolution, several arguments can be made. Although the four types appear to be equally efficient from the two previous points of view, it is obvious that type I leaves the genome more exposed to possible damage by physical/chemical mutagenic agents. Types II, III and IV differ mainly in the extent of chromatin compaction achieved. Substitution of histone by PL (and removal of the otherwise unnecessary nucleosomes) and finally by P leads to an increasingly more compacted nucleus that may have finally been selected by the constraints imposed by the mechanisms of fertilization [40] or by other selective pressure mechanisms yet to be established.

Evolution of the nuclear sperm-specific proteins

From what has been discussed in the previous sections, it is clear that the PL-type represents an intermediate type both from the structural and functional point of view. Whereas protamines have only been found at the tips of the most evolved groups from both the protostome and deuterostome branches, PL proteins are already found in the eukaryote groups preceding this branching. A rather exhaustive analysis recently carried out in our laboratory on several organisms belonging to different groups of the phylum Cnidaria has revealed that a primitive H1-PL-I protein is the major chromosomal protein found in the sperm nucleus of these organisms (unpublished results, but see Fig. 4A). Evidently the intermediate structural features of the PL proteins, discussed earlier, must reflect their intermediate evolutionary position.

All this, provides support for the early hypothesis put forward by SUBIRANA several years ago that sperm-specific nuclear proteins, including protamines, may have evolved from a common histone ancestor [64]. In the light of the experimental information presently available, we propose the evolutionary pathway which is shown in Fig. 5A. Accordingly, all the chromosomal sperm

Source : MNHN, Paris

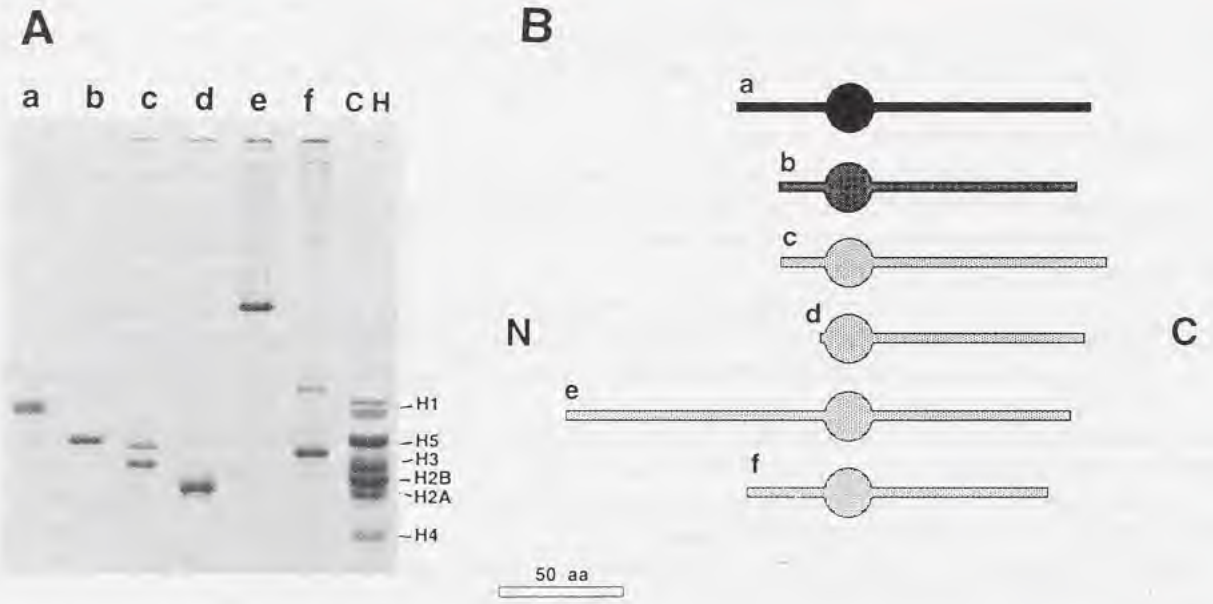

FIG. 4. — Widespread distribution of PL-I proteins in the eukaryotic kingdom. **A:** Urea acetic acid polyacrylamide gel electrophoresis of (a) calf thymus somatic histone H1, (b) chicken erythrocyte histone H5, (c-f) nuclear proteins from the sperm of (c) *Mullus surmuletus* (fish), (d) *Chelysoma productum* (tunicate), (e) *Spisula solidissima* (clam) and (f) *Metridium senile* (anemone). (The arrows point to the PL-I protein components.) **B:** Comparative schematic representation of the teriary structure (circle = globular core, linear regions = N, C terminal domains) of the protein PL-I from different organisms and somatic histones H1, H5. The letter symbols designate the same species as in (A). CH = chicken erythrocyte histone standard.

proteins would have arisen from a primitive histone precursor, presumably the same from which the somatic lysine-rich histone H1 lineage would have also originated. The enormous structural variability and the rapid evolution [37] of the proteins from the H1 family make it very difficult to trace the origin of such a precursor and its identification with any protein previous to the metazoan organization (see KASINSKY, this volume).

The next step in the evolution of these proteins in the case of the sperm lineage would involve an increase in the arginine contents of this originally lysine-rich precursor. In the early stages such protein would still coexist with a full complement of the somatic-type of histones (histone H type of spermiogenesis, see Fig. 1B). The sperm-specific H1 histones of the sperm of echinoderms provide a good example of such histones. A further increase in the arginine content would allow these molecules to displace and replace the somatic histones, as occurs with the PL-I molecules found in the sperm of many bivalve molluscs [2, 4, 5, 13]. It is important to point out here that PL-I is not only restricted to molluscs. As shown in Fig. 4A, PL-I proteins have been identified in many phylogenetic groups, including chordates [59] and vertebrates [61].

In the next evolutionary step the PL-I proteins would lose their globular core, giving rise to smaller PL proteins either by processes of post-translational cleavage (Fig. 5B), such as in *Mytilus* [21], or by other mechanisms yet to be described, such as alternative splicing. Evidence for alternative splicing has already been provided for the bovine protamine 2 gene [41].

The small PL proteins may have then evolved into the arginine-rich protamines, which are found in the upper phylogenetic levels of the deuterostome and protostome branches. In the process of selection, arginine may have been selected over lysine because of its higher potential to form hydrogen bonds [11].

The evolutionary pathway from histones to specialized sperm-specific histone H1, PL-I, PL

Source : MNHN, Paris

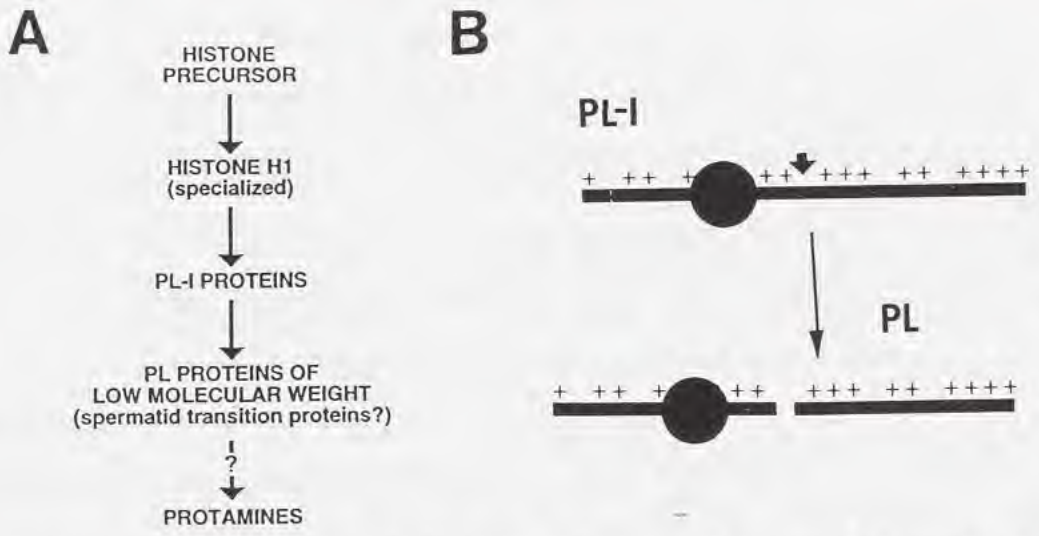

FIG. 5. — **A:** Proposed evolutionary relationship for the nuclear sperm-specific proteins. **B:** Some of the low molecular weight PL proteins (Mr < 15000 Da) might have arisen from post-translational cleavage of a larger PL-I precursor [21].

and protamines appears in repeated instances within different phyla both in protostomes [69] and in deuterostomes [60]. The presence of the most primitive protein types, H and PL, decreases as the P type increases in the most evolved groups of the phylogenetic tree. Thus, the scheme shown in Fig. 5A represents, in fact, the "mode" rather than the "tempo" [30] of evolution followed by the chromosomal sperm proteins.

The selection of an H1-related protein type at the base of this evolutionary pattern in each different phylum may have occurred initially by a process of evolutionary convergence due to the intrinsic ability of this molecule to condense chromatin and possibly to lock it in an "inert" structure. This could have happened at the expense of increasing the arginine content of this protein and/or the stoichiometric ratio of this molecule with respect to the nucleosome subunit. In this process the tripartite organization of histone H1 and most of the secondary structure of its globular core have been significantly conserved and therefore both functional and structural convergence most likely occurred in this case. At the other end of this evolutionary pattern, all protamines exhibit a highly arginine-rich composition with very similar primary sequences consisting of arginine clusters (see KASINSKY, in this volume). The possibility therefore exists that this could represent a convincing case of genuine sequence convergence [29] starting from different PL-I proteins. It should be pointed out, however, that such a possibility is hard to prove considering the small size (30-80 amino acids) of protamines, which excludes the possibility of long range cladistic analysis [28].

The evolutionary "mode" of the nuclear sperm-specific proteins presented here represents an alternative to the "retroviral hypothesis" proposed for the origin of protamines [38]. This hypothesis was initially put forward in order to account for the sporadic distribution of the protein types H and P in fishes. As a matter of fact, a recent thorough reexamination of the distribution of the three protein types H, PL, and P in the sperm of bony fish [61] has not been able to provide any support to the retroviral origin of protamines in this vertebrate class.

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Source : MNHN, Paris

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Evolution and Origins of Sperm Nuclear Basic Proteins

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ABSTRACT

Although the DNA-binding, basic nuclear proteins of sperm (SNBPs) are highly diverse, constraints on their diversity in particular taxa suggest that they may play an adaptive role in such taxa and are not merely randomly distributed. For example, in the subphylum Vertebrata, internally fertilizing tetrapods have arginine-rich protamines or keratinous protamines that condense sperm chromatin to a greater extent than in externally fertilizing frogs or fish having sperm histones. In such taxa "R-CLUES," *r*elatively *c*onstant *l*argest *u*nits of *e*volutionary *s*imilarity, are broader phylogenetically for mammals (for example, infraclass Eutheria) than for frogs (for example, genus *Bufo*). The evolution of SNBPs appears to be saltatory rather than continuous. Consequently, the phylogenetic breadth of R-CLUES may assist in the classification of particular taxa where SNBPs are either protamine-like or histone-like amongst particular genera of frogs (*Xenopus* versus *Silurana*) or subfamilies/families of fish (Salmoninae versus Sparidae). SUBIRANA & COLOM (1987), AUSIO *et al.* (1987), and CHIVA *et al.* (1991) have proposed that protamines in molluscs may have evolved from very lysine-rich H1 histones. This is supported by the presence of protamine-like SNBPs with trypsin-resistant cores in cnidarian sperm. The presence of protamine-like SNBPs in lower plants may be a case of convergent evolution. Highly condensed chromosomes in the eukaryotic dinoflagellate *Crypthecodinium cohnii* and the highly condensed nucleoid in the bacterium *Chlamydia trachomatis*, both have lysine-rich basic proteins similar to portions of histones H5 or H1, respectively. This suggests possible additional cases of convergent evolution amongst basic proteins of condensed chromatin preceding the origin of the primitive type of sperm in metazoans.

RÉSUMÉ

Évolution et origines des protéines basiques nucléaires des spermatozoïdes

Bien que les protéines nucléaires basiques liées à l'ADN des spermatozoïdes (PNBS) soient extrêmement diversifiées, les contraintes pesant sur leur diversité dans des taxons particuliers suggèrent qu'elles pourraient jouer un rôle adaptatif dans de tels taxons et qu'elles ne sont pas simplement distribuées au hasard. Par exemple, dans le sous-embranchement Vertebrata, les Tétrapodes à fécondation interne ont des protamines riches en arginine ou des protamines kératineuses qui condensent plus intensément la chromatine du spermatozoïde que chez les Amphibiens ou les Poissons à fécondation externe possédant des histones dans le spermatozoïde. Dans de tels taxons, les plus grandes unités relativement constantes de similarité évolutive ("R-CLUES" en Anglais) ont une plus grande étendue phylogénique chez les Mammifères (par exemple, l'infraclasse Eutheria) que chez les Amphibiens (par exemple, le genre *Bufo*). L'évolution des PNBS apparaît saltatoire plutôt que continue. De ce fait, l'ampleur phylogénique des "R-CLUES" peut être utile pour la classification de taxons particuliers chez lesquels les PNBS sont proches des protamines ou proches des histones, parmi des genres particuliers d'Amphibiens (*Xenopus* versus *Silurana*) ou des familles ou sous-familles de Poissons (Salmoninae versus Sparidae). SUBIRANA & COLOM (1987), AUSIO *et al.* (1987), et CHIVA *et al.* (1991) ont proposé que les protamines chez les Mollusques aient évolué à partir d'histones H1 très riches en lysine. Cette hypothèse est confortée par la présence de PNBS proches des protamines avec des domaines résistants à la trypsine dans les spermatozoïdes des Cnidaires. La présence de PNBS chez les plantes inférieures pourrait être un cas d'évolution convergente. Les chromosomes hautement condensés du

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Dinoflagellé Eucaryote *Crypthecodinium cohnii* et les nucléoïdes très condensés de la Bactérie *Chlamydia trachomatis* ont tous deux des protéines basiques riches en lysine, respectivement similaires à des portions des histones H5 et H1. Ceci suggère l'existence de cas supplémentaires d'évolution convergente parmi les protéines basiques de la chromatine condensée, antérieurs à l'apparition du type primitif de spermatozoïde chez les Métazoaires.

Sperm basic proteins (SNBPs) that bind to DNA in animals and plants are highly diverse [12], in sharp contrast to the evolutionarily conservative nucleosomal histones that characterize all other cell types. SNBPs range from low molecular weight arginine-rich protamines in which these basic residues are clustered repeatedly, as in the sperm nucleus of the cartilaginous fish *Scyliorhinus canicula* [17], to replacement by a sperm-specific variant of histone H1, as in the frog *Rana ridibunda* [34]. In sperm of the goldfish *Carassius auratus* [40], the entire complement of histones is retained along with the nucleosomal organization of chromatin. Keratinous protamines with disulfide bonds also can be found in the sperm of *Scyliorhinus* [17]. Some protamine-like SNBPs show similarities to very lysine-rich histones, as in bivalves like the surf clam *Spisula solidissima* [7] and the mussel *Mytilus edulis* [8]. Other protamine-like SNBPs have intermediate compositions containing lysine and sometimes histidine along with arginine, as in the turtle *Chrysemys picta* [20], some frogs like *Ascaphus truei* [33] and *Bufo japonicus* [60], and in some bony fish, including sticklebacks like *Pungitius pungitius* [37]. Some crab species have no basic proteins at all in their sperm [12].

DISCUSSION

Internal fertilization as a constraint on SNBP diversity

Is this diversity of SNBP type due to randomness or adaptation? In BLOCH's classical paper he came down on the side of the former when he stated, "It is proposed that the variability (non-conservatism) of the protein reflects an evolutionary indifference to a relatively unimportant protein in an inert nucleus" [12, p. 107]. In the same paper, he also indicated that "... although a phylogenetic relationship is often apparent from the similarity of proteins within tightly defined taxonomic groups (e.g. , the clupeids, or the eutheria), there seems to be no evolutionary trend. Most of the classes of sperm proteins are represented within most of the broad taxa." [12, p. 99]. In fact, analyses [30-32, 53] of the distribution of SNBPs in animals indicates (Fig. 1, left) that the mode of fertilization acts as a constraint on SNBP diversity, such that either protamines, keratinous protamines or protamine-like SNBPs are present in sperm of internally fertilizating taxa. Thus, sperm of the honey bee *Apis mellifera* [5] and the barnacle *Balanus nubilus* [21] are not exceptions to this rule, as thought by BLOCH [12], but appear to contain protamine-like SNBPs upon gel electrophoresis, while cytochemical analysis [53] indicates the presence of protamine-like SNBPs in sperm of the platyhelminth *Notoplana* and the nematode *Thelastoma periplaneticola*. The only exception to this rule known to date is the deep sea bony fish *Cataetyx laticeps* (order Ophidiiformes, family Bythitidae). Recently, SAPERAS *et al.* (58) have discovered that internally fertilizing sperm of this species contain histones and an additional sperm-specific protein that is compositionally similar to erythrocyte H5 from grass carp. Perhaps this anomaly is a consequence of the fact that internally fertilizing deep sea fish such as brotulas have relatively short larval stages [41, p. 469], a reproductive strategy designed to enable a small number of young to settle in suitable habitats in their benthic environment. Such a progenetic tendency [39] in this viviparous fish might require the utilization of histone-like genes, rather than the more usual protamine-like genes to condense DNA, as the first sperm might have to be made after fewer cell divisions following fertilization in such a precociously maturing organism [35]. It may be possible to test this experimentally by examining the relationship between SNBP type and progenesis in the five species of bythids that are confined to shallower freshwater or weak brackish water environments [41, p. 226].

Source : MNHN, Paris

Internal fertilization as a constraint on SNBP diversity is most clearly observed in the tetrapods. In the subphylum Vertebrata (Fig. 1, right), internally fertilizing eutherian mammals possess keratinous protamines [10, 42], metatherians have non-keratinous P1-like SNBPs [63], birds contain protamines [19, 42] and reptiles have protamine-like SNBPs [20, 30]. In some frogs with external fertilization, like *Rana*, sperm-specific histones H1 and H2B are present in the nucleus. Here [50], sperm chromatin is not as tightly condensed as in amniote species with protamines or protamine-like SNBPs. Thus, there is a trend in the tetrapods from variability of SNBP type in externally fertilizing anurans to relative constancy of protamine type in internally fertilizing urodeles and amniotes [30, 31]. This can also be observed in cartilaginous fish [17, 30] and in bony fish [12, 30], with the sole exception of *Cataetyx laticeps* [58] noted above. In molluscs [18, 23], internal fertilization also constrains SNBP diversity in mesogastropods, neogastropods and cephalopods. Here protamine-like SNBPs or keratinous protamines are found.

R-CLUES as phylogenetic measures of SNBP diversity.

If we define a new term, "R-CLUES," as the "*r*elatively *c*onstant, *l*argest *u*nits of *e*volutionary *s*imilarity [32], then amniotes have phylogenetically broader R-CLUES than externally fertilizing vertebrates like anurans and most bony fish by virtue of the constraint of internal fertilization. For example, Table 1 shows that similar P1 keratinous protamines occur throughout the *infraclass* Eutheria, but similar protamine-like intermediate SNBPs are confined to particular *genera* of Australian frogs, like *Litoria* [33], and stickleback fish, such as *Gasterosteus* [37].

The acronym "R-CLUES" is also intended to denote the search for "clues" for "R," the arginine content of SNBPs in particular taxa. Thus, a low arginine content of 4.5 mole percent in the lysine-rich protamine PL-IV of the mussel *Mytilus edulis* [8] is typical for R-CLUES at the family level (Mytilidae) in the class Bivalvia. In the genus *Bufo*, R-CLUES for these representatives of the order Anura are delineated by a much higher arginine composition, for example 42.3 mole percent, in *Bufo japonicus* protamine P1 [60].

Is internal fertilization the only constraint on SNBP diversity in animal sperm? Apparently not. As seen in Table 1, echinoderms are all external fertilizers. Constancy in the marine environment probably accounts for the relative constancy of SNBP type in this taxon. All of the echinoderms have sperm-specific histone H1 [44], with R-CLUES showing variation for different orders [30]. In externally fertilizing tunicates, the constant marine environment also appears to maintain the relative constancy of SNBPs. A protamine-like P1 SNBP that resembles H1 histone is the principle protein for all species studied thus far [22, 56], with some variation for R- CLUES for the genus *Styela*. Therefore, from R-CLUES indicated in Table 1, we see that internal fertilization is a particular kind of constraint for certain taxa on land, like amniotes, and in the marine environment, like mesogastropods, neogastropods, cephalopods and cartilaginous fish. I agree with Saperas [55, p. 330] that internal fertilization tends to fix the type of SNBP, insofar as the more specialized the biology of reproduction, the more such variation impacts negatively on SNBP function.

As can be seen in Fig. 1 and Table 1, the evolution of SNBPs appears to be saltatory rather than continuous, in the sense that these proteins can differ quite markedly between related taxa. In the case of eutherian mammals, P1 keratinous protamines are sufficiently similar to constitute a family of related proteins [17], yet they are amongst the most rapidly diverging polypeptides studied [47], evolving at rates close to that of fibrinopeptides. Nevertheless, these proteins can easily be distinguished from the protamines of metatherian mammals [63] and the arginine-rich protamines of birds [19, 42], both of which lack cysteine.

Source : MNHN, Paris

TABLE 1. — R-CLUES of representative animal taxa.

[1], Classification modified from [11, 28, 41, 65]. [2], See legend of Fig. 1 for definitions of P, KP, PL and H. H1 = sperm-specific histone H1, with higher arginine content than somatic histone H1. H2B = sperm - specific histone H2B. [3], R-CLUES (*r*elatively *c*onstant *l*argest *u*nits of *e*volutionary *s*imilarity) are in italics. [4], Int. = internal; Ext. = external. [5], Ter. = terrestrial; Mar. = marine; FW = freshwater. [6], P1, P2a, P2b, = keratinous protamines. [7], Reference. [8], Taxonomic ranking as follows: phylum > subphylum > class > infraclass > superorder > order > suborder > family > subfamily > genus [11, 28, 41]. [9], *Silurana* = proposed genus [14] to contain former species *Xenopus tropicalis* (2n=20) and *X. epitropicalis* (2n=40). [10], Some primitive urodele species are external fertilizers [30]. [11], Some species are anadromous; i.e., marine, but breed in freshwater [37]. [12], *Ascidia* is a facultative internal fertilizer [22] in this suborder. [13], Includes SNBPs from 3 of the 15 living families of suborder Lacertilia (Sauria) but from only one family (Colubridae) of 15 in suborder Serpentes [65]. [14], In order Carcharhiniformes. May also include SNBPs of *Squalus acanthias* [30], family Squalidae, order Squaliformes and R-CLUES may therefore be broader phylogenetically than indicated here. [15], May also include SNBPs of *Sepia officinalis* [23] in order Sepoidea.

Taxon[1]	SNBP Type[2], Reference	R-CLUES[3]	Fertilization [4]	Habitat[5]
Phylum Chordata				
Subphylum Vertebrata				
Class Mammalia	KP(P1)[6] [10, 42][7]	*Infraclass*[8] Eutheria	Int.	Ter., Mar.
	KP (P1+P2a+P2b)[6] [47]	*Order* Primates	Int.	Ter.
	PL [63]	*Order* Marsupalia	Int.	Ter.
Class Aves	P [19, 42]	*Superorder* Paleognathae	Int.	Ter.
Class Reptilia	PL [30]	*Order* Squamata[13]	Int.	Ter.
	PL [20]	*Order* Testudines	Int.	Ter.
Class Amphibia	PL [30]	*Order* Caudata	Int., Ext.[10]	Ter.
	PL [14, 30]	*Genus Xenopus+Silurana*[9]	Ext.	Ter.
	PL [34, 60]	*Genus Bufo*	Ext.	Ter.
	PL [33]	*Genus Litoria*	Ext.	Ter.
	H1 [1, 34]	*Genus Rana*	Ext.	Ter.
Class Osteichthyes				
Division Teleostei	PL [37]	*Genus Gasterosteus*	Ext.	Mar., FW[11]
	P [42]	*Subfamily* Salmoninae	Ext.	Mar.
	H [58]	*Family* Sparidae	Ext.	Mar.
Class Chondrichthyes				
Subclass Elasmobranchii	KP, P [17, 43]	*Family* Scyliorhinidae[14]	Int.	Mar.
Subphylum Urochordata	PL (P1) [22]	*Suborder* Phlebobranchiata	Ext.[12]	Mar.
	PL (P1, P2) [22, 56]	*Genus* Styelidae	Ext.	Mar.
Phylum Echinodermata				
Class Holothuroidea	H1, φo [45]	*Genus Holothuria*	Ext.	Mar.
Class Echinoidea	H1,H2B [30, 44]	*Order* Echinoida	Ext.	Mar.
Subclass Asteroidea	H1 [44]	*Order* Forcipulata	Ext.	Mar.
Phylum Mollusca				
Class Polyplacophora	PL (P1, P2) [7, 23]	*Genus Mopalia*	Ext.	Mar.
Class Gastropoda				
Order Archaeogastropoda	PL (P2) [18, 23]	*Family* Trochidae	Ext.	Mar.
Order Patellogastropoda	PL (P1) [18, 23]	*Family* Lottiidae	Ext.	Mar.
Order Mesogastropoda	PL (P3) [18, 23]	*Family* Littorinidae	Int.	Mar.
Order Neogastropoda	PL (P3) [18, 23]	*Family* Nucellidae	Int.	Mar.
Class Bivalvia				
Subclass Pteriomorphia	PL [23]	*Family* Mytilidae	Ext.	Mar.
Subclass Heterodonta	PL [23]	*Family* Tridacnidae	Ext.	Mar.
Class Cephalopoda	P [23]	*Order* Teuthodea[15]	Int.	Mar.

Source : MNHN, Paris

Amongst the vertebrates, the saltatory nature of SNBP evolution can best be seen in frogs and bony fish. Table 1 shows that different genera of frogs can have either histone-like SNBPs or arginine-rich protamine-like SNBPs, whereas some bony fish can show a similar alteration at the familial level (subfamily Salmoninae versus family Sparidae). This alteration appears to be of a sporadic nature [58]. Analysis of such R-CLUES may assist us in determining whether protamine → histone or histone → protamine transitions may have occurred in closely related taxa. The clearest example we have to date of the success of such an analysis is the ability of R-CLUES to distinguish between frogs of the genus *Xenopus* [61] and morphologically similar frogs of the genus *Silurana* [14]. In the polyploid genus *Xenopus*, there is a lineage of frogs with diploid chromosome numbers of 36 → 72 → 108 that includes *laevis* and another lineage with 20 → 40 diploid chromosome numbers that includes *tropicalis* and *epitropicalis*. Only SNBP type can clearly distinguish these two lineages biochemically. Thus, R- CLUES can distinguish two separate genera, since the former lineage has intermediate type, protamine-like SNBPs, while the 20 → 40 line contains only histones, with one additional spermatid/sperm-specific protein [38].

Recently, on the basis of morphological criteria, CANNATELLA & TRUEB [14] have split the genus *Xenopus* and have placed the discordant *tropicalis* and *epitropicalis* species into the genus *Silurana*, which they have resurrected from GRAY (1864). Thus, amongst the five genera of pipid frogs [65, p. 364], the two species of *Silurana* are more closely related to *Hymenochirus* and *Pipa* than to species of *Xenopus*. As we predicted the electrophoretic profile of *Silurana epitropicalis* SNBPs solely on the basis of chromosome number before doing the actual experiment [38], it appears that R-CLUES may be useful characters for systematic studies, along with more traditional analyses.

Origins of SNBPs.

What is the origin of the SNBP pattern in the deuterostomes? Recently, SAPERAS *et al.* [57] presented two models, both of which take into account the difference between histones present in echinoderm sperm and protamine-like SNBPs in urochordates and the cephalochordate *Branchiostoma floridae*. The lamprey *Petromyzon marinus* also has somatic-like histones in its sperm [57]. However, cartilaginous fish have protamines and keratinous protamines [17]. Alternation between protamines and histones can be seen in different families of bony fish and frogs, whereas urodeles and the amniotes have only protamines, protamine-like SNBPs or keratinous protamines.

From these data, SAPERAS *et al.* [57] concluded that both models would have histones at the base of the SNBP pattern in deuterostomes. However, examination of Fig. 1 (left) indicates that at the root of deuterostome phylogeny are taxa that show protamine-like SNBPs, either by cytochemical criteria, as in nematodes and platyhelminths [53], or by a combination of cytochemical and biochemical analysis, as in cnidaria [6, 53]. In the case of the sea anemone *Metridium senile*, AUSIO [4] has shown that the SNBP belongs to the PL-I type, related to H1 histones, with a peptide core that is trypsin resistant. It would seem, therefore, that another model could place protamine-like SNBPs at the base of deuterostome phylogeny, with reversions to histones occurring in particular taxa like echinoderms and agnatha. This might be due to the loss of protamine genes or gene expression in deuterostome evolution [57], or perhaps due to shifts in developmental timing, such that an earlier onset of spermiogenesis in particular taxa might require selection for histone rather than protamine gene expression [35]. Such an argument was made in the previous section for the lack of protamines in the internally fertilizing deep sea fish *Cataetyx laticeps* [58]. Perhaps it might also apply to the histone-like SNBPs in sessile echinoderms evolved from motile ancestors [11, p. 838].

What might be the origins of protamine-like SNBPs in the lower metazoa? SUBIRANA & COLOM [59], AUSIO *et al.* [9] and CHIVA *et al.* [18] have proposed that protamines in molluscs

Source : MNHN, Paris

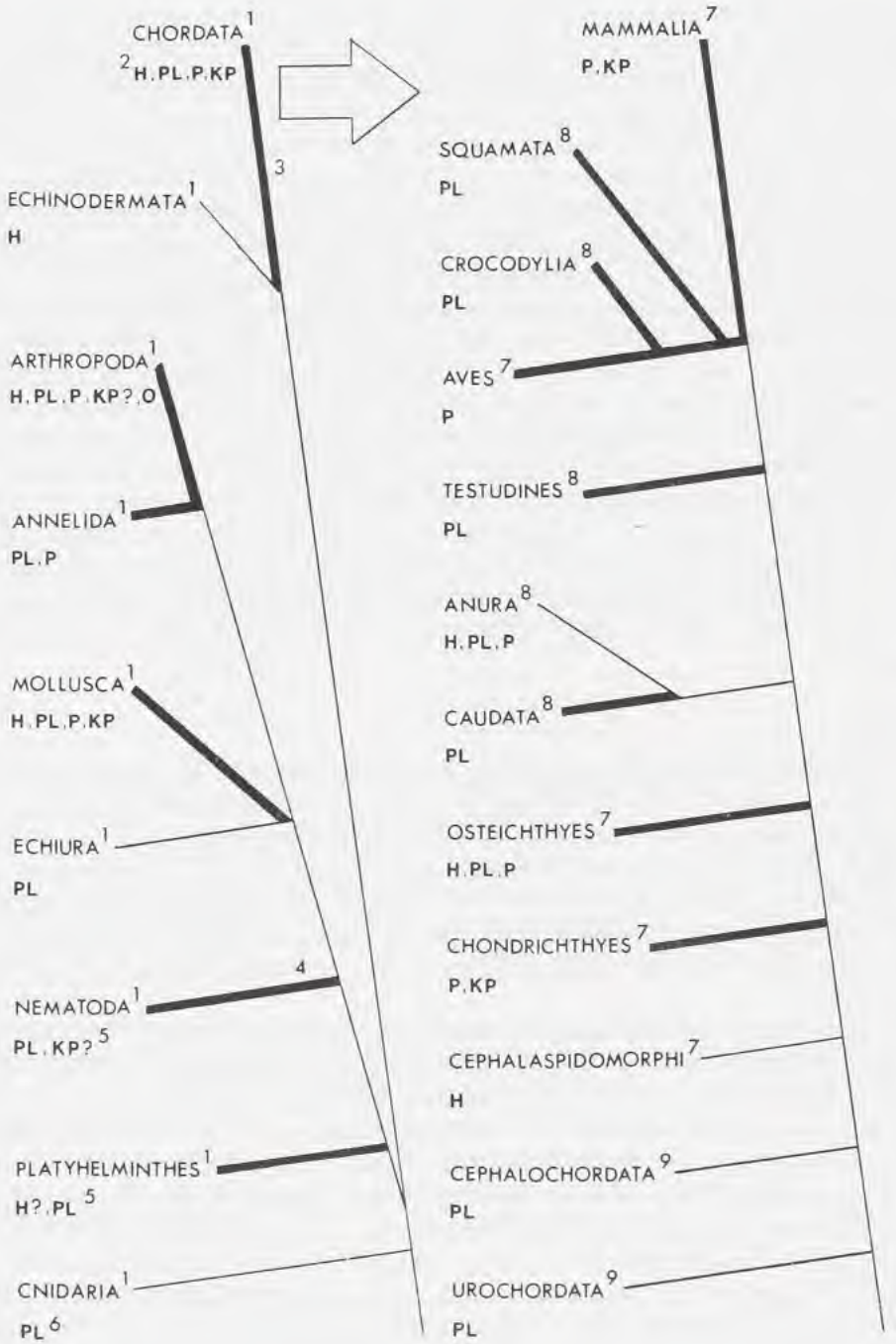

FIG. 1. — Diversity of SNBPs in animals. (Cladogram on left has been modified from [13, pp. 873, 882] and on right modified from [28, pp. 607, 628, 669, 684, 707, 736]. 1, Phylum; 2, SNBP type: H = somatic like-histones; PL = intermediate protamine-like SNBPs; P = protamines; KP = keratinous protamines; 0 = no SNBPs present in sperm nucleus; 3, Thick line indicates some or all species in this taxon are internally fertilizing; 4, Position of Nematoda is not indicated in [13, p. 882]; 5, SNBP type based on cytochemical data [12, 53] or 6, on electrophoretic and cytochemical data [6, 53]; 7, Class; 8, Order; 9, Subphylum.

Source : MNHN, Paris

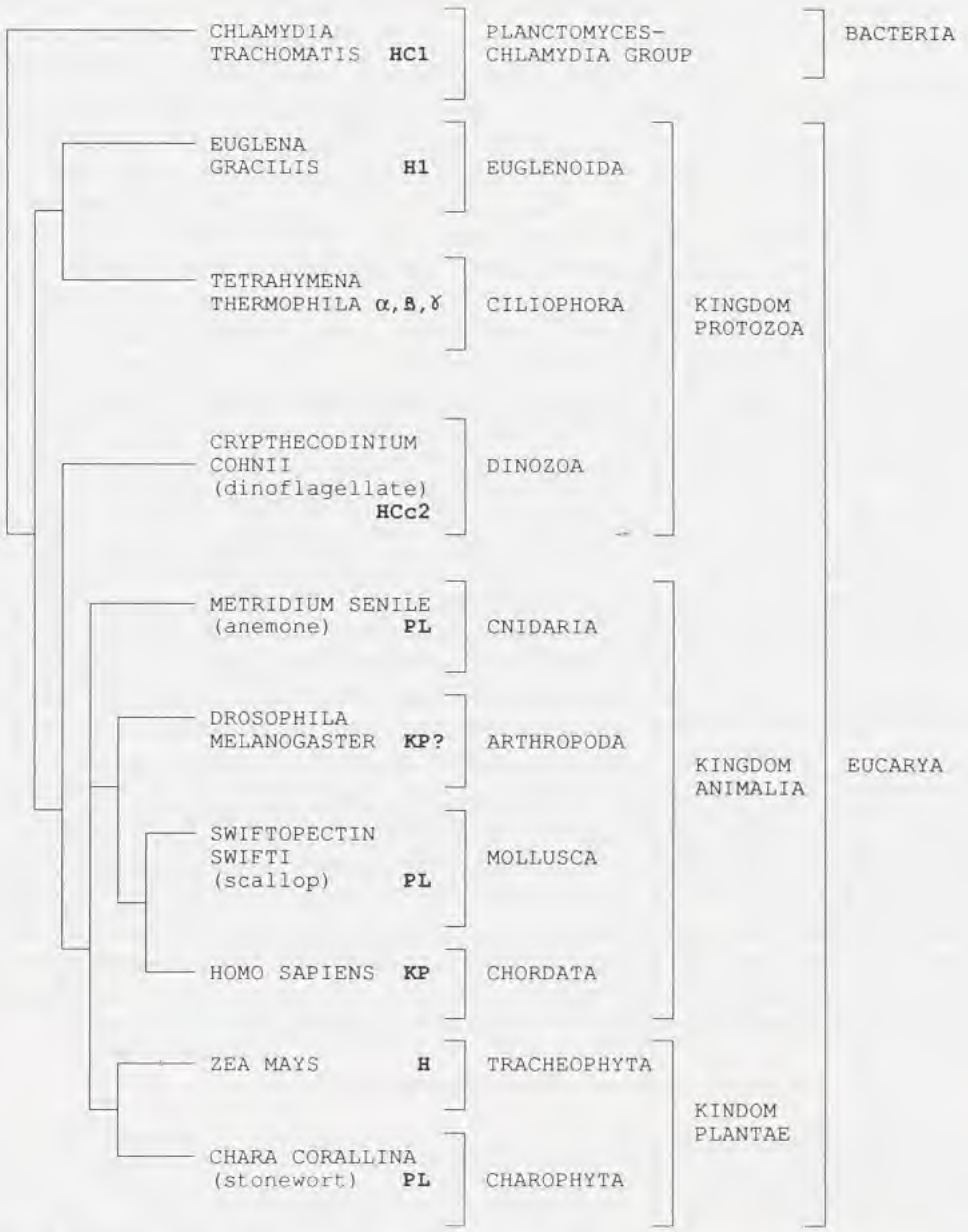

FIG. 2. — Possible outgroups of animal SNBPs amongst nuclear basic proteins of plant sperm and protists. Cladogram is modified from CAVALIER-SMITH's 18s rRNA phylogeny for 150 eukaryotes [16] and WOESE's 16s rRNA phylogeny for bacteria [64].

may have arisen from very lysine-rich H1 histones by the evolutionary route H1 histones → protamine-like SNBPs → protamines. The presence of PL-I basic proteins in cnidarians [4, 6] supports such an evolutionary pathway. Also, as can be seen in Fig. 2, plant SNBPs [51] provide an outgroup for patterns of SNBP diversity in animal phyla since they arose from different protistan ancestors than did animals. Therefore, the presence of protamine-like SNBPs in biflagellate, motile sperm of green algae, such as the stonewort *Chara corallina* [49], and in bryophytes, like the liverwort *Marchantia polymorpha* [48], along with the presence of H1-like histones in condensed nuclei of the nonmotile male gamete in a higher plant such as *Lilium*

Source : MNHN, Paris

longiflorum [62], indicates that the histone H1→ protamine-like SNBP transition may be a case of convergent evolution [24]. This attests to the importance of the connection between sperm motility and the condensation of sperm chromatin in the origin of SNBPs, a connection that is further emphasized if we consider the nature of the basic proteins in highly condensed nuclei of chromosomes in protists [46, p.10]. Thus, the eukaryotic dinoflagellate *Crypthecodinium cohnii* [54], which has condensed chromosomes, lacks histones and nucleosomes but contains two lysine-rich base proteins, one of which, HCc2, shows 38% of residues identical with the 53 residues near the carboxyl terminus of the erythrocyte-specific histone H5 of duck. More primitive dinoflagellates have histones, whereas in *Noctiluca* [15], a transitional form, histones are present in the giant vegetative cells, but not in the small sexual swarmer stages. As CAVALIER-SMITH [15, p. 350] points out, "It could be that histone loss in the noctilucean swarmers is adaptive, analogous to the replacement of histones by protamines or other basic proteins in many animal sperm to allow more compact sperm nuclei." The histone H1 in nuclei with condensed chromatin in *Euglena gracilis* [29] is unique and in the condensed, transcriptionally inactive, germ-line micronuclei of *Tetrahymena thermophila*, [2], proteolytic processing of histone H1 gives rise to three H1-like polypeptides, α, β and γ. GOROVSKY [26] notes that "like some other transcriptionally inert nuclei such as nucleated red blood cells [25] and some histone containing sperm [1], micronuclei have different linker-associated (H1-type) histones than their transcriptionally active counterparts." RAIKOV [46, p. 71] calls the micronuclei of many Ciliophora "spermal" as they "are filled with compact chromatin, just as the nuclei of spermatozoa are." In this regard it is interesting to note that the highly condensed nucleoid in the elementary body of the prokaryotic bacterium *Chlamydia trachomatis* [27] contains a basic protein, Hc1, that shows 34.9% identity in a 106-amino acid overlap with histone H1 of the sea urchin *Lytechinus pictus*. This is highly unusual for bacteria, however, where the nucleoid is normally not so highly condensed [52] but may none the less contain some H1-like basic proteins as in *Pseudomonas aeruginosa* [36], or protamine-like basic proteins as in *Escherichia coli* [3].

From the viewpoint of SNBP types in lower metazoans, there is a sense of déjà vu with regard to the role of H1-like histone and related basic proteins in condensing chromatin in some protists and a bacterium. Perhaps it is the common need for tight packing of chromatin in motile sperm of animals and lower plants and in the condensed nuclei or chromosomes of motile protists that has given rise, probably by convergent evolution, to some common features within the diverse spectrum of basic proteins binding to DNA in these different organisms.

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Source : MNHN, Paris

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Source : MNHN, Paris

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Source : MNHN, Paris

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Male Germ Line Specific Histones of Sea Urchins and Sea Stars

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ABSTRACT

Sea urchin sperm nuclei contain two classes of histone molecules specific to the male germ line: Sp H1 and Sp H2B. Sea stars have only Sp H1. These molecules contain repeated tetrapeptide motifs of serine–proline adjacent to two basic amino acids in domains which are absent from somatic histones. The tetrapeptides are always phosphorylated except in mature sperm nuclei, where they are correlated with unusual physical properties of the chromatin including high packing densities and long nucleosomal repeat lengths. The occurrence of these molecules in the echinoderms, their modulations during spermatogenesis and following fertilization, their modes of interaction with DNA, and speculations concerning their functions are discussed.

RÉSUMÉ

Les histones spécifiques de la lignée germinale mâle des Oursins et Étoiles de Mer

Les noyaux des spermatozoïdes des Oursins contiennent deux classes de molécules d'histones spécifiques de la lignée germinale mâle: Sp H1 et Sp H2B. Les Étoiles de mer ont seulement Sp H1. Ces molécules contiennent des motifs répétés de tétrapeptides constitués de sérine-proline adjacentes à deux acides aminés basiques, dans des domaines qui sont absents dans les histones somatiques. Les tétrapeptides sont toujours phosphorylés sauf dans les noyaux des spermatozoïdes mûrs, où ils sont corrélés avec des propriétés physiques inhabituelles de la chromatine telles que de hautes densités et de grandes longueurs de répétition nucléosomiques. La discussion porte sur la présence de ces molécules dans les Échinodermes, leur modulation pendant la spermatogenèse et après la fécondation, leurs modes d'interaction avec l'ADN et des hypothèses sur leurs fonctions.

The minimal eukaryotic chromosome consists of DNA and its associated basic proteins, the histones. A histone octamer, in association with 146 bp of DNA, makes up the core nucleosome. The core contains two molecules each of the H2A, H2B, H3 and H4 histones. The remaining histone, H1, is almost always present and is associated with an additional ~20 bp of DNA to form two DNA loops about the protein core. H1 is also associated with a variable amount of linker DNA between adjacent cores. The variable linker gives rise to chromatin of different average nucleosomal repeat lengths.

The chromatin fiber may be folded into a solenoid of ~30 nm diameter or into other fiber structures. Several higher orders of packing result in the formation of highly condensed mitotic chromosomes or even more highly condensed sperm chromatin. Knowledge of these latter steps of chromatin packaging is extremely limited.

POCCIA, D., 1995. — Male germ line specific histones of sea urchins and sea stars. *In*: JAMIESON, B. G. M., AUSIO, J., & JUSTINE, J.-L. (eds), Advances in Spermatozoal Phylogeny and Taxonomy. *Mém. Mus. natn. Hist. nat.*, **166** : 475-489. Paris ISBN : 2-85653-225-X.

Source : MNHN, Paris

Histones are present in virtually all cell types of all eukaryotes. The histones are distinguished by a high degree of evolutionary conservation of amino acid sequence. For example, H4 of calf and pea differ by two conservative amino acid substitutions out of 102. H3 and H4 are the most conserved, H2A and H2B less so, and H1 the most divergent. This high degree of conservation implies that histone structure is critical for proper assembly of nucleosomes and interaction with trans–acting factors affecting gene expression.

Not all nuclei contain histones. Among the earliest characterized chromatin proteins were the protamines by MIESCHER in 1897 [31]. The protamines are more basic than the histones and are not simply related to them in sequence. Their evolutionary relationship to the histones has been discussed [82, 25, see AUSIO in this volume].

MIESCHER's source of tissue was mature salmon sperm. It is now clear that sperm nuclear proteins in general are free from the evolutionary constraints operating on somatic cell histones, and the variety of non–histone structural proteins now known to be associated with sperm DNA seems at least as great as the variety of shapes that sperm display (for reviews see [25, 42]). The reason that these constraints do not apply may be that, once spermiogenesis is well underway, neither transcription, replication, nuclear transport nor mitosis have any longer to be performed. Instead, the main task is packaging of sperm DNA into a very condensed mass to be transported to the egg. It follows that in the most extreme cases of substitution of histones by other nuclear structural proteins during spermatogenesis, the transitions should take place late in spermiogenesis, after normal nuclear function ceases.

Unlike salmonids or mammals, the sea urchins and sea stars retain histones in their mature sperm cells and there are no traces of protamines or protamine–like molecules. In the urchins, however, two of the five histones, Sp H1 and Sp H2B, differ from their somatic counterparts in ways discussed below. Similar Sp H1s (but not H2Bs) are found in sea star sperm. Sp histones first appear in sea urchin spermatogenesis in the spermatogonia/spermatocytes where they completely replace somatic histones of their classes. Thus unlike protamines, the non–conserved, testis–specific echinoderm histones must function in typical nuclear processes.

In mature sperm, an additional role of Sp histones appears to be to confer unusual physical properties upon the chromatin, in particular a very large amount of linker DNA which, for the sea urchin, gives rise to the longest nucleosomal repeat lengths known, and also a high degree of chromatin compaction. How this is accomplished and speculations on the structure and function of these proteins is the subject of this article.

RESULTS AND OBSERVATIONS

Occurrence of Sp histones among echinoderms

Sp histones have been well–documented for sea urchins, established for a few sea stars and may be lacking in sea cucumbers. Ophiouroids and crinoids have to my knowledge not been investigated.

The time of radiation of the echinoderm classes including the echinoids, asteroids and holothuroids may have been about 500–600 million years ago [32, 39, 55, 63, 64]. Resolution of class relationships between echinoids, asteroids and holothuroids is not yet clear owing to difficulties in resolving estimates based on different methods and a poor fossil record for the time of their divergence [64].

All extant echinoids stem from a member of the order Cidaroida, *Myocidaris* [63]. The cidaroids or "primitive sea urchins" diverged from the euechinoids or "modern" urchins about 200 million years ago. Sp histones have been found by sequence or electrophoretic analysis in all species of sea urchin so far examined including the cidaroid *Eucidaris tribuloides* [81]. *Eucidaris* Sp H1 is about 9 amino acids shorter than that of the euechinoid *Strongylocentrotus purpuratus* H1 owing to a shorter C–terminal region. Their Sp H2Bs are of similar same size (see Table 1).

Source : MNHN, Paris

TABLE 1. — Relative sizes of Sp H1 variants. Sizes given in number of amino acid residues.

Species	Known Size [71]	Calculated Size [81]
Sea Urchin		
Parechinus angulosus	248	
Strongylocentrotus purpuratus		248 ±1.4
Eucidaris tribuloides		239 ± 0.8
Lytechinus pictus		248 ± 1.6
Lytechinus variegatus		238 ± 2.0
Arbacia punctulata		243 ± 2.8
Sea Star		
Patiria miniata (1)		251 ± 4.0
Patiria miniata (2)		260 ± 4.5

Sea star sperm (*Aphelasterias japonica, Patiria miniata, Asterias vulgaris*) lack large Sp H2B subtypes, but have large Sp H1 molecules only one of which has been sequenced [16, 30, 81, 86]. Sea cucumber (*Holothuria tubulosa*) sperm chromatin possesses somatic type histones, a sperm specific H1, and a 78–amino acid molecule resembling the C–terminal region of somatic H1s called ϕ_0 [3, 4]. The sequence of the sperm–specific H1 is not known, and therefore its relationship to Sp H1s is uncertain.

Sp histones in vivo

That sperm–specific histones of the H1 and H2B classes were present in mature sea urchin sperm was recognized with the advent of gel electrophoretic analysis [11, 37, 38, 72]. The single Sp H1 and the two or three variants of Sp H2B appeared larger than their somatic counterparts. A decade later, the first amino acid sequences of Sp histones were determined for *Parechinus angulosus* [68–71]. These demonstrated that the larger size was due mainly to N–terminal extensions on both Sp H1 and Sp H2B (and a smaller C–terminal extension on Sp H1). The N–terminal regions are rich in serine, suggesting the potential of multiple phosphorylation sites *in vivo*. In addition, Sp H1 also had a long alanine–lysine–rich region preceding the C–terminal domain (Fig 1). As discussed below, Sp histones at first approximation can be considered chimeric, a relatively conserved histone core altered by additions at the termini.

Subsequently it was found that of the five histone classes, only the Sp histones were modified in male pronuclei immediately after fertilization [17, 44, 48, 49]. These modifications were at least in part due to phosphorylation events which were mapped to serines on the N–terminal regions of Sp H2B and the N– and C–terminal regions of Sp H1 [17]. Thus sperm–specific histones are specifically phosphorylated following fertilization. Subsequently they are either displaced or diluted by histones from the egg during the first few cell divisions of the embryo [49, 59].

The origin of the Sp histones during sea urchin spermatogenesis was also investigated. Expression of the Sp H2B gene occurs in the earliest cells of the male germ lineage [51, 81]. This pattern of expression is different from that of a gene product utilized exclusively by the mature sea urchin sperm such as bindin, a major acrosomal constituent, whose mRNA is detected late in spermatogenesis [33]. This also contrasts with the expression of protamine genes in fish and

Source : MNHN, Paris

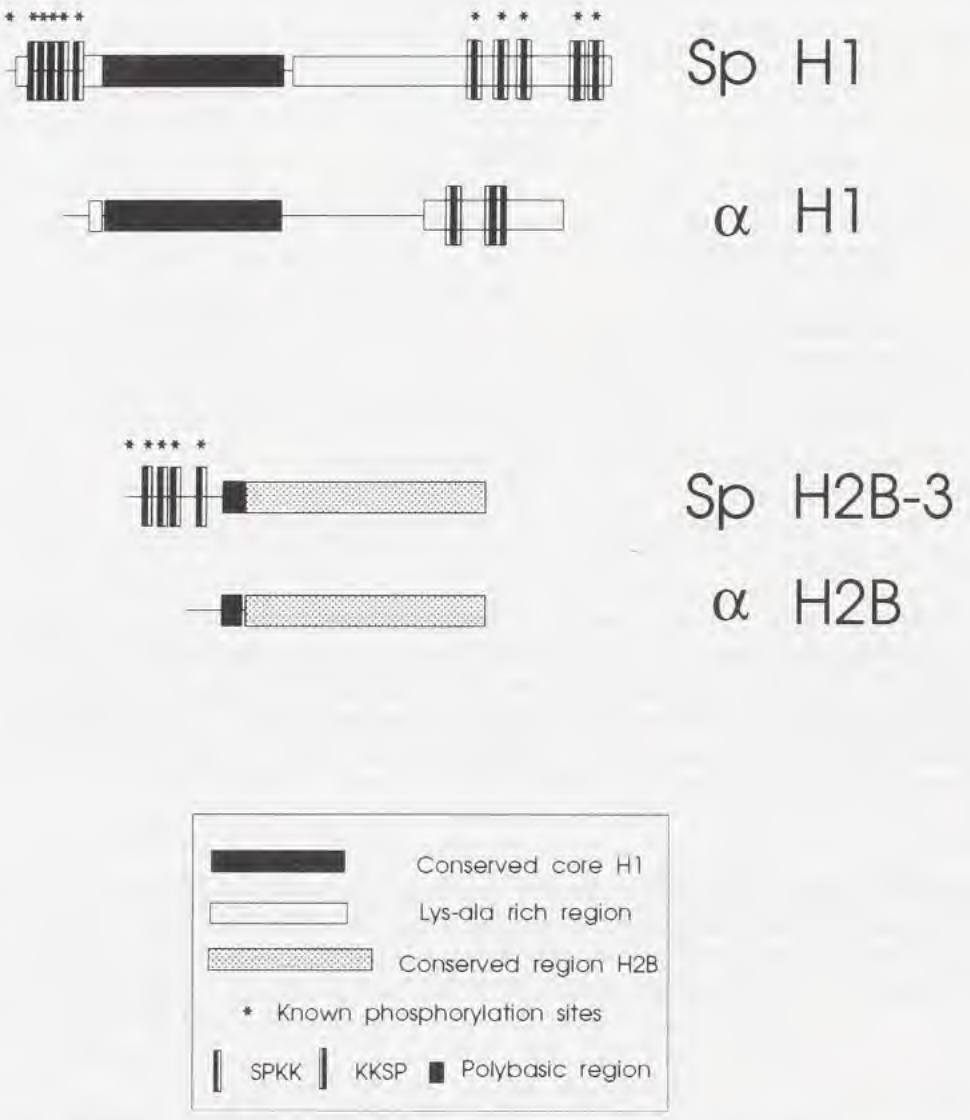

FIG. 1. — Diagram of regions in sea urchin histone variants: Comparison of Sp and somatic histones.
Derived from sequences in the Protein Identification Resource database (accession numbers: Sp H1 A02586, Sp H2B–3 A02620) and in the GenBank/EMBL database (accession numbers: embryo somatic histones H1 α, J01171, H2B α V01144).

protamines and transition protein genes in mammals which are expressed post–meiotically in spermatids [34, 46]. The cells showing expression of Sp H2B are the spermatogonia and pre–replicative spermatocytes, cells undergoing DNA synthesis. Indeed, the structure of the Sp H2B gene suggests a cell–cycle regulated gene typical of somatic histones [51].

Protamine mRNAs are long–lived and their expression is translationally controlled, the proteins appearing several developmental stages after their messages are transcribed [34, 46]. However, during sea urchin spermatogenesis, the Sp histones appear in the earliest cells of the male germ line (spermatogonia/spermatocytes) suggesting they are under transcriptional control [50]. From their first appearance until the transition from late spermatid to spermatozoon, Sp histones are phosphorylated [50]. The major phosphorylation sites in *S. purpuratus* gonadal

Source : MNHN, Paris

histones were mapped to serines in the N–terminal regions of the Sp H1 and Sp H2B [50]. All sites of phosphorylation were mapped in more detail using spermatid chromatin of *E. esculentus* [23] and shown to occur on consensus amino acid sequences SPKK (see below). As shown in Fig. 1, Sp H1 has six of these sites on serines of the N–terminal region and three sites on serines of the C–terminal region, only one of which was not in an authentic SPKK site (at SPQK). In Sp H2B, the four sites were located in the N–terminal region, one of which is at the related sequence SPSK [23].

From the parallel behavior of Sp H1 and Sp H2B phosphorylation during spermatogenesis and following fertilization, it was proposed that lack of phosphate in these histones was correlated with extreme compaction and that phosphorylation permitted the molecules to function by decreasing the affinity of the histone arms for DNA allowing the more conserved regions to dominate function [17, 43, 50]. It was suggested that the unphosphorylated arms serve to cross–link the chromatin into a compact form and stabilize it [17, 36, 82] and that this function is only exerted in the mature sperm.

The identity of the kinase responsible for SPKK phosphorylation is uncertain. Clearly it must act rapidly following fertilization. However, no reports of an endogenous sperm kinase with SPKK specificity have appeared. Eggs contain kinases which will phosphorylate the SPKK regions [14, 53, 54, 77]. SPKK sites themselves are related to cdc2 kinase sites, and are almost always present in multiple copies in C–terminal regions of somatic H1s, suggesting that they may be modified by mitotic kinases. On the other hand, inhibition of male pronuclear decondensation by amounts of the drug 6–DMAP which are sufficient to inhibit cdc2 kinase during mitosis in the same cells has little or no effect on the phosphorylation of Sp histones after fertilization [52], suggesting that the enzyme is different from cdc2, or cdc2 is differentially regulated immediately after fertilization (during G1 of the sea urchin cell cycle). Kinases of the male germ line responsible for Sp histone phosphorylation during spermatogenesis or phosphatases responsible for dephosphorylation in late spermatids are as yet uncharacterized.

Whether phosphorylation is required for removal of Sp histones from male pronuclear chromatin is not known. A protease which cleaves Sp H1 has been isolated from sea urchin eggs and suggested to be involved is such removal [76]. Its specificity for SPKK sequences is not unequivocal however, and in addition there is no evidence for proteolytic degradation of Sp H1 taking place on chromatin *in vivo*.

Primary and secondary structure of Sp histones

Sea urchin Sp H1 can be divided into four domains (Fig. 1), a conserved globular domain characteristic of all H1 molecules (amino acids 40–114), a long region rich in alanine and lysine (120–185) , and the N– and C–terminal regions characterized by multiple tetrapeptides of serine–proline adjacent to two basic amino acids (lysine and/or arginine) [43], a motif which has become known as SPKK [73].

The Sp H2B molecules (present as two or three variants in each species) can be regarded as having a well conserved C–terminal region, a 40 amino acid N–terminal region containing, in Sp H2B–1, several SPKK repeats in the first ~20 residues and a glycine–rich stretch in the next 20 amino acids followed by a conserved polybasic region rich in arginine [43, 82].

Thus the Sp H1 and Sp H2B molecules may be considered chimeric. The N–terminal regions of both and the C–terminal region of Sp H1 are unique domains related in sequence to one another which are "added" to a more conserved core molecule. The long lysine–arginine motif of Sp H1 is present in other H1s where it is shorter, may contain proline and is punctuated by SPKK motifs. Its length and lack of proline, though, make the long lysine–arginine region a unique feature of Sp H1.

Source : MNHN, Paris

TABLE 2. — Repeating tetrapeptides in Sp H1 and Sp H2b.
$X_7 = X_1X_2$SPQKR where X_1 = P or A; X_2 = G or A.
"SPKK" sequences in bold. Sea star sequence from [30]. For original references to sea urchin sequences, see [45].

Histone	Species	N–Terminal sequence							
Sp H1	*S. purpuratus*	(X7)AA	**SPRR**	**SPKK**	**SPRK**	**SPKK**		**SPRK**	RS
Sp H1	*P. angulosus*	(X7)AA	**SPRK**	**SPRK**	**SPKK**	**SPRK**	ASA	**SPRR**	KA
Sp H1	*E. crassa*	(X7)AA	**SPRK**	**SPRK**	**SPRK**	**SPKK**	K??	**SPRK**	R?
Sp H1	*S. granulosus*	(X7)AA	**SPRK**	**SPKR** G	**SPKK**	**SP??**			
Sp H1	*S. nudus*	(X7)AA	**SPRR**	**SPKK**	**SPRR**	**SPKK**	KSS	**SPRR**	K
Sp H1	*P. miliaris*	(X7)AA	**SPRK**	**SPRK**	**SPKK**	**SPRK**	ASA	**SPRX**	K
Sp H1	*E. esculentus*	(X7)AA	**SPRK**	**SPRK**	**SPKK**	**SPRK**	ASA	**SPRR**	
Sp H1	*A. vulgaris* (Sea star)	AG	**SPRK** P	**SPKK**					
Sp H2B (1)	*S. purpuratus*	PSQRSPT	**KRSP** T	**KRSP** Q					
Sp H2B (1)	*P. miliaris*	PRSKSPT	**KRSP** T	**KRSP** Q					
Sp H2B (1)	*P. angulosus*	PRSKSPT	**KRSP** T	**KRSP** T	**KRSP** Q				
Sp H2B (1)	*E. esculentus*	PSQKSPT	**KRSP** T	**KRSP** Q					
Sp H2B (2)	*S. purpuratus*	PRSPSKT	**SPRK** G	**SPRR** G	**SPSRK** A	**SPKR** G			
Sp H2B (2)	*P. miliaris*	PKSPSKS	**SPRK** G	**SPRK** G	**SPRK** G	**SPRK** G			
Sp H2B (2)	*L. pictus*	PRSPSKS	**SPRK** G	**SPRK** G	**SPRK** G	**SPRK** G			
Sp H2B (2)	*P. angulosus*	PRSPAKT	**SPRK** G	**SPRK** G	**SPSRK** A	**SPKR** G			
Sp H2B (2)	*E. esculentus*	PKSPSKS	**SPRK** G	**SPRK** A	**SPKR** G				
Sp H2B (2)	*E. crassa*	PKSPSKG	**SPRK** G	**SPRK** G	**SPTRR** G				
Sp H2B (3)	*P. angulosus*	PRSPAKT	**SPRK** G	**SPRK** G	**SPRK** G	**SPSRK** A	**SPKR** G		
Sp H2B (3)	*E. esculentus*	PRSPSKS	**SPKK** G	**SPRK** A	**SPKR** GGK				

Few sequences are available for sea star sperm histones. The complete sequence of H2B from *Asterias rubens* sperm indicates a single variant lacking in SPKK repeats [29] consistent with electrophoretic data indicating lack of sea star Sp H2Bs. Sequencing of the N–terminal region of *Asterias vulgaris* Sp H1 however shows at least two SPKK motifs are present [30] separated by a proline (Table 2). Since the partial sequence includes only the first 11 residues, additional SPKKs may be present.

The repeating amino acid sequences of Sp histones were originally described as tetrapeptides for Sp H1 and pentapeptides for Sp H2Bs [68–71]. The two basic amino acids may lie to either side of the serine–proline motif in different variants. Therefore the tetrapeptide serine–proline adjacent to two basic amino acids is the minimal common pattern which incorporates the repeated sequences present in Sp H1 and all of the Sp H2B's known (Table 2) [17, 43]. This grouping emphasizes the placement of a phosphorylatable serine near two basic amino acids. SPKK, considered a subset of SPXX, is a unit of predicted secondary structure [43] in which serine may interact with amino acids at positions 3 and 4 [73]. The nature of the third and fourth residues is not important as their interactions do not involve their side chains. The SPXX is not always coincident with SPKK in Sp histones (Table 2). The N–terminal domains containing multiple SPKK's in all Sp histones are predicted as β–turn structures [17, 36, 43, 73] or extended kinked helices [70].

Source : MNHN, Paris

In an early comparison of β–turn structures in a variety of proteins, 24 of 30 phosphorylated residues from 14 different proteins were found in β–turn regions [62]. As pointed out above this is the case *in vivo* for SPKK regions of Sp histones which are both phosphorylated and predicted β–turns. The four residues forming β–turns are often found at the surface of globular proteins and greatly affect the conformation and specificity of antibody–combining sites and enzyme active sites. They can be considered to represent cassettes which can be inserted by evolution or genetic engineering to alter the geometry of proteins [24]. SPKK "cassettes" are found in multiple copies in a variety of nucleic acid binding proteins [47].

The tetrapeptide containing region at the N–terminus of Sp H2Bs is not its only region predicted to have β–turn structure. The glycine–rich stretch between the SPKK repeats and the polybasic region of Sp H2Bs is also predicted to have β–turn structure which therefore dominates the first 40 residues of Sp H2B–1[43].

SPKK tetrapeptides have been modelled by analogy with the nucleic acid binding drugs neotropsin and Hoechst 33258 [73] and it has been suggested that they form β–turns stabilized by hydrogen bonds between the OH and CO groups of serine and the NH amide groups of the third and fourth amino acids of the tetrapeptide. More recently, a σ–turn structure has been proposed to co–exist as a conformational isomer with the β–turn in Sp H1 [78].

Measurements in solution (see below) are consistent with β–turn structure, but ultimately the question of secondary structure must be settled by crystallization of the protein both bound to and free of DNA. The only crystal structure of an H1 histone domain solved so far, for the globular region of erythrocyte H5, is a helix–turn–helix structure likely to exist in the most conserved domain of Sp H1 as well [57].

The two tetrapeptides included in the peptide SPRKSPRK are equivalent turn structures in exchange with a more extended structure by NMR analysis in solutions of DMSO [78]. The peptide may exist in a conformation between a β–turn and a σ–turn. In the latter, the amides of the two serine residues are further away and fit binding to the minor groove of DNA less well. Model building has not solved unequivocally the problem of how the binding occurs to DNA.

The long lysine–alanine stretch in Sp H1, free of proline, is predicted [43, 80] and found 15, 21] to have a high α–helical content. It has been suggested that the long α–helical stretch accommodates the extra linker in sperm DNA [21]. The long linker has been proposed to form three equivalent superhelical turns per repeat, optimizing sperm chromatin packing efficiency in some way [22]. Alternatively, the lysine–arginine–rich region can be considered a palindrome and has been modeled as two α–helices stabilizing an extra superhelical turn [75]. This reverse loop or figure–eight solenoid model would force the linker DNA into a right–handed superhelical loop towards the centre of the solenoid structure.

Interaction of Sp histones and DNA.

Most evidence supports the idea that Sp histones bind to linker DNA and that the N–terminal regions prefer the minor groove of AT–rich regions. H1 histones are known to bind to linker DNA, but Sp H2B may behave similarly. Linker binding was originally suggested on the basis of indirect evidence [17, 86], but more recent measurements confirm the proposal. Reductive methylation of lysines after their exposure following dissociation from linker DNA is consistent with binding of Sp H2B through its N–terminal region [20]. Crosslinking of DNA to Sp H2B histone also demonstrates linker binding [7]. Sp H2B in hybrid reconstituted nucleosomes protects an additional 8 bp of linker DNA against micrococcal nuclease digestion in a more efficient way than wheat H2A, which has a similar extension but lacks SPKK motifs [28].

Binding of Sp histones to linker DNA may involve short–range or long–range, intrastrand or interstrand, interactions. Although evidence for cross–linking of chromatin fibers *in vivo* by Sp histones is lacking, many studies demonstrate the unique abilities of Sp histones to bind strongly to DNA resulting in aggregation. For example, Sp H1 is more effective at aggregating

Source : MNHN, Paris

superhelical DNA than somatic H1s such as calf thymus histone [36]. In reconstitution of SV40 DNA with core nucleosomes, at intermediate ionic strengths, sea urchin core histones form large intermolecular associations which have been attributed to the Sp H2B [35].

Current data favor a model of Sp histone binding to the minor groove of AT–rich regions of DNA. Based upon binding competition studies of the N–terminal peptide of Sp H1 and Hoechst 33258, it was proposed that Sp H1 binds to the minor groove [73], although others have found no evidence for competitive inhibition [19]. Further support for binding to AT–rich regions was obtained by hydroxyl radical footprinting [8] using a synthetic peptide of two SPKK's. For a more detailed discussion of this subject, see [9].

Binding of DNA to the synthetic octapeptide SPKKSPKK conjugated to anilino–acridine was studied using DNase 1, OH–radical footprinting and osmium tetroxide. AT–selectivity of binding supported a minor groove binding model [5] and was interpreted as SPKK recognition of defined DNA sequences. Although a single tetrapeptide could unwind and extend DNA and distort the double helix, two SPKKs seem to be minimally necessary for the AT–specific minor groove binding [13].

Conformational changes in DNA have been noted upon binding of the octapeptide SPKKSPKK. This peptide without the acridine binds to calf thymus DNA or various polynucleotides. As assessed by circular dichroism, only binding to poly (dA–dT)•poly(dA–dT) induces major changes in the spectrum consistent with a φ–type condensation pattern [6]. Other peptides containing TPKK alter DNA condensation giving φ–type CD spectrum and it was concluded that the H1β–turn, although it becomes more rigid, remains a β–turn upon DNA binding [12].

What role, if any, selectivity for AT–rich regions plays in sperm chromatin structure is not clear. The stoichiometry of histone–DNA binding *in vivo* implies that Sp histones bind to all the nuclear DNA and it would be surprising if binding is not essentially uniform.

Effect of Sp histone phosphorylation on DNA binding.

Phosphorylation of the serine of the SPKK repeat should have two important consequences for its DNA binding. First the resultant addition of two negative charges per repeat should effectively neutralize the entire structure thus drastically lowering its DNA affinity. Second, by disrupting the serine hydroxyl group, the phosphate should interfere with the secondary structure.

Fewer studies have been made of the interactions of phosphorylated Sp histones with DNA than with the unphosphorylated forms. But some direct measurements support the concept of weaker binding of phosphorylated N–terminal regions to DNA. Intermolecular migration of Sp H1 which involves dissociation from the DNA is facilitated by phosphorylation [23]. Phosphorylated Sp H1 binds less tightly to DNA by affinity column chromatography [23, 73]. Multiple phosphorylation in the N–terminal but not the C–terminal regions of Sp H1 drastically reduces DNA affinity [23]. Purified N–terminal domains of Sp H1 from sperm and male germ cells were compared by several criteria including DNA cellulose binding, salt induced precipitation, thermal denaturation of DNA and inhibition of HO 33258 binding which showed that binding to DNA was always weaker for the phosphorylated peptides [19]. Unphosphorylated Sp H1 induces salt–dependent folding of long oligonucleosomes and promotes DNA self–association more effectively than phosphorylated [23].

Since sea urchin sperm chromatin has the longest repeat length known, it has been suggested that Sp H1 and Sp H2B arms might bind to and neutralize linker DNA [17, 18, 86] as discussed above. The correlation of long linker DNA in sperm chromatin with dephosphorylation and shorter linker in spermatogenic or pronuclear chromatin with phosphorylation suggested that the arms of the Sp histones might bind to linker only when their phosphates were removed [17, 18, 60]. When unphosphorylated Sp histones are bound to linker, the DNA is more resistant to

Source : MNHN, Paris

micrococcal nuclease digestion than either pronuclear [60] or spermatid [18] chromatin linkers which may be bound weakly if at all by the N–terminal arms.

Sp histones and the structure of sperm chromatin

Sperm nuclei containing unphosphorylated Sp histones have several properties which may be dependent upon these special nuclear proteins. These include high chromatin compaction, the shape of the nucleus, and the exceptional amount of linker DNA of the chromatin.

Sea urchin sperm heads are generally conical; sea star and sea cucumber spherical. Thus conical sperm nuclear shape in the echinoderms is correlated with the presence of Sp H2B, occurring only in the sea urchin. The transition from spherical to conical morphology occurs in the late spermatid, when dephosphorylation of Sp histones takes place, and the sea urchin late spermatid resembles the sea star mature sperm in nuclear shape. However, it is not clear whether nuclear shape is dependent upon dephosphorylated Sp H2B.

Sea cucumber sperm chromatin is not as highly condensed as sea urchin or sea star chromatin [40, 41]. Thus the highest degrees of chromatin compaction occur in the sea urchin and sea stars which contain a Sp H1, but again the relevance of this has not been demonstrated. *In vitro*, oligonucleosomes from sea urchin sperm seem to be more compact compared to somatic chromatins by sedimentation criteria [83]. In addition, circular dichroism measurements indicate that sea urchin sperm chromatin behaves uniquely with respect to compactness as a function of ionic strength [58].

It is impossible at present to compare in detail the higher order structure of chromatin in the nuclei of echinoderm sperm because preparation artifacts and current methodology do not permit. Sea star nuclei show regular ordered structures in minimally disrupted nuclei by electron microscopy [16]. Possibly related higher order structures have been reported in sea urchins as well [87, 88]. Chromatin fibre diameters vary between species but these are apparently not correlated with repeat length [1, 65, 85].

Whatever the exact structure of chromatin in the mature sperm nuclei, certain correlations can be made with the degree of compaction and Sp histone phosphorylation. Sea urchin sperm chromatin is packed about as densely as mitotic chromosomes [17]. Phosphorylation of Sp histones occurs prior to decondensation of the chromatin following fertilization [17]. If decondensation of chromatin is blocked by the kinase inhibitor 6DMAP, Sp histones appear to be normally phosphorylated [52]. This indicates that phosphorylation is not sufficient to induce decondensation, although it might still be necessary. Likewise it suggests that, in spite of the similarity in target sequences, the Sp kinase is not the cdc2 mitotic kinase which is inhibited under the same conditions.

The nucleosomal repeat lengths of sea star and sea cucumber sperm chromatins are shorter than sea urchin but longer than typical somatic chromatin repeat lengths (Table 3) [10, 86]. Repeat lengths increase in the sea urchin at the last stages of spermatogenesis when Sp histones are dephosphorylated [18]. In contrast, repeat lengths are constant during sea cucumber spermatogenesis [10]. Sea star and sea cucumber sperm chromatins each have repeat lengths approximately 20 base pairs (bp) shorter than sea urchin [10, 86]. Analysis of sperm chromatin of crinoids and ophiuroids is unavailable.

Thus the longest nucleosomal repeat lengths are correlated with the presence of both Sp H2B and Sp H1. Given data demonstrating that both bind to linker DNA, this is not surprising. However, sea cucumber sperm nucleosomal repeat lengths are about the same as those of sea stars. We do not know enough about the chemistry of the special sperm H1 and ϕ_o in sea cucumber to be able to speculate about their relationship to long linker.

It is not obvious whether the long linker is a consequence of Sp histone binding or is controlled by some other factor. Sea urchin sperm H1 is more efficient than somatic H1s at

Source : MNHN, Paris

TABLE 3. — Average nucleosomal repeat lengths of echinoderm sperm chromatins

Group	Species	Repeat Length (bp)	Reference
Sea Urchin			
	Eucidaris tribuloides	238 ± 2	[81]
	Strongylocentrotus purpuratus	249 ± 5; 250 ± 12	[81, 27]
	Lytechinus pictus	245 ± 5	[81]
	Arbacia lixula	241	[66]
	Strongylocentrotus intermedius	237 ± 5	[86]
	Arbacia puntulata	260 ± 26	[26]
Sea Star			
	Patiria miniata	225 ± 3; 222± 5	[81, 16]
	Aphelasterias japonica	224 ± 6	[86]
Sea Cucumber			
	Holothuria tubulosa	228 ± 5	[10]
	Thyone briareus	233	[84]

organizing nucleosomes at low packing densities in reconstitution systems *in vitro* using polyglutamic acid and poly d(A–T)•poly d(A–T) [67]. Sperm core histones (containing Sp H2Bs) are likewise more efficient at being recruited [67]. In this assay, Sp H1 gives repeat lengths approximately the same as those *in vivo*. It would be of great interest to determine if phosphorylation of Sp histones shortens the nucleosomal repeat length, using *in vitro* chromatin reconstitution systems.

DISCUSSION

Phylogenetic trees for core histones have recently been constructed [79]. The H2A/H2B histones which form dimers appear to have co–evolved and are 10 times more divergent than the H3/H4 pair. Sea urchin Sp H2B was the only divergent core histone without a correspondingly divergent H2A. It was suggested that sea urchin Sp H2Bs evolved more rapidly than other sea urchin H2Bs or H2As and that they perform a function that is atypical or assemble nucleosomes in a different way free of typical constraints. Based on the discussion above, it seems likely that Sp H2B function in the unphosphorylated state is atypical, namely the packaging of sperm chromatin, but that it can assemble proper nucleosomes when in the phosphorylated state when its conserved regions dominate its function.

It has been suggested that the repeating structure of Sp H2B is related to the structure of vertebrate protamines based upon (1) the similarity of basic residue spacing between proline and hydroxyamino acids and the nature of the basic residues (arginines) when the repeat is considered a pentapeptide of the form pro–X–X–X–ser (with two of three X's basic), and (2) the similar size of protamines and the Sp H2B N–terminal extension [82]. In this view vertebrate protamine genes would have evolved from the N–terminal region of Sp H2B toward greater basicity and more efficient DNA packaging.

However, similar motifs are not unknown among invertebrates. For example, sperm–specific protein ϕ3 from the mussel *Mytilus californianus* has two SPKK motifs punctuating an otherwise largely lysine–alanine–rich sequence [2]. This molecule of 5 kD is predominantly α–helical in structure and in some ways resembles the C–terminal region of Sp H1. In what way should we imagine its evolutionary relationship to sea urchin Sp histones?

Source : MNHN, Paris

The scheme proposed by AUSIO in this volume takes a different approach to the origin of protamines. It postulates a primitive histone precursor giving rise to somatic H1s and sperm-specific basic nuclear proteins. Echinoderm Sp histones would result from an increase in arginine content of the precursor and would co–exist with histones, whereas in other organisms intermediate type protamine–like (PL) molecules would evolve and replace somatic histones. By loss of their globular cores, these would become small PL molecules as found for example in *Mytilus*. The small PLs might then evolve to protamines. This scheme would, however, have to take into account the appearance of similar SPKK domains on the N–termini of both Sp H1s and Sp H2Bs and the possibility of their assembly from scattered SPKK domains seen in somatic H1s remains.

Another problem concerns speculation on evolutionary and functional relationships merely on the basis of amino acid sequence similarities. A good example is the central globular domain of H1 histones which is highly conserved. The crystal structure of this domain of the H1 variant H5 from erythrocytes was recently solved to 2.5 Å resolution [56, 57, 61]. Its secondary structure is remarkably similar to a liver–specific transcription factor HNF–3γ in a helix–turn–helix motif. However, sequence similarity between the two proteins is limited (12% of the structurally equivalent residues). Each is a member of a large family of proteins highly conserved in sequence and function. The choice of whether these proteins have diverged from a common ancestor or converged to a common three–dimensional structure is uncertain.

The presence of very long nucleosomal repeat lengths and Sp histone molecules in sperm of echinoids and asteroids suggests that these related molecular patterns were developed by a common ancestor and conserved to the present day. From the functional standpoint, it is not obvious why such long repeats are useful, except to allow binding of the extensions of the Sp histones. That this binding may allow a special kind of compaction of chromatin in these organisms has been discussed above. Certainly in a functional sense, the arms of Sp histones might be considered to lend protamine–like qualities to the Sp histones.

The motif SPKK or its variants (threonine instead of serine, arginine instead of lysine) is always present in the C–terminal domain of somatic H1 molecules and could represent a cassette borrowed by other molecules which would function in strong but reversible nucleic acid binding. The occurrence of the multiple tetrapeptides in a variety of DNA and RNA binding proteins has been noted [47]. It has also been argued that the related SPXX motif is found often in gene regulatory proteins [74]. Yet unfortunately too few data are available for comparisons to be made among the sperm histones of echinoderms to generalize about similarities of function of evolutionary relationships.

Since sperm cells compete for successful fertilization, they can be under great selective pressure for the brief period following spawning. This may depend on events occurring before fertilization, such as local environmental conditions, or after, such as the ability of the egg to rapidly disassemble and reassemble male chromatin. It would seem likely that efficient packaging of the sperm nucleus is an advantage in marine environments for motile sperm. However, co–evolution of egg mechanisms for formation of the male pronucleus from the sperm nucleus following fertilization may limit the rate at which sperm nuclear structural proteins can evolve.

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Source : MNHN, Paris

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Source : MNHN, Paris

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Source : MNHN, Paris

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The gene encoding the sperm-specific basic nuclear protein ϕ_o from sea cucumber

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ABSTRACT

The gene encoding the sperm-specific protein ϕ_o from the sea cucumber *Holothuria tubulosa* has been cloned and characterized. Sea cucumber sperm chromatin displays a somatic-like histone complement and is beaded with a constant 227 bp DNA linker length, second to the longest repeat of sea urchin chromatin. Protein ϕ_o, a small basic protein reminiscent of the C-terminal tail of histone H1, appears at the onset of spermiogenesis and accumulates in ripe sperm. The ϕ_o gene displays a coding frame interrupted by three large intervening sequences which combine to make it the longest for a sperm-specific protein yet reported (*ca.* 17.7 kb). The identified gene is present as a single copy and, in turn, encodes a polyadenylated transcript. The protein ϕ_o specified by this gene has 77 residues, reproducing unaltered the partial amino acid sequence of the protein previously determined. The structural arrangement and content of the ϕ_o gene are totally unrelated to cell-cycle regulated histone gene structure. Instead, it combines several features common to replication-independent genes coding for histone variants and even to protamine genes. Inference is made about the potential implications of this divergence in gene arrangement as regards chromatin transitions and modulation of gene activity occurring during spermiogenesis.

RÉSUMÉ

Le gène codant pour la protéine basique nucléaire ϕ_o spécifique des spermatozoïdes chez l'holothurie

Le gène codant pour la protéine basique nucléaire ϕ_o spécifique des spermatozoïdes chez l'holothurie *Holothuria tubulosa* a été cloné et caractérisé. La chromatine du spermatozoïde d'holothurie présente un complément d'histone de type somatique et forme un chapelet avec un intervalle constant de 227 paires de bases d'ADN, qui est la seconde plus longue répétition de chromatine d'échinoderme. La protéine ϕ_o, une petite protéine basique qui rappelle l'extrémité C-terminale de l'histone H1, apparaît au début de la spermiogenèse et s'accumule dans les spermatozoïdes mûrs. Le gène ϕ_o comprend une région codante interrompue par trois grandes séquences intercalaires qui se combinent pour en faire le gène le plus long connu actuellement pour une protéine spécifique des spermatozoïdes (environ 17.7 kb). Le gène identifié est présent en copie unique et code pour un transcrit polyadénylé. La protéine ϕ_o spécifiée par ce gène a 77 résidus parmi lesquels on retrouve sans erreurs la séquence partielle d'acides aminés précédemment déterminée pour la protéine. L'organisation structurale et le contenu du gène ϕ_o sont sans relations avec la structure des gènes des histones régulés par le cycle cellulaire. Au contraire, ce gène combine plusieurs caractéristiques communes aux gènes indépendants de la réplication codant pour des variants d'histones et même aux gènes de protamines. La conséquence potentielle de cette divergence dans l'organisation des gènes en ce qui concerne les transitions de la chromatine et la modulation de l'activité du gène pendant la spermiogenèse est discutée.

PRATS, E. & CORNUDELLA, L., 1995. — The gene encoding the sperm-specific basic nuclear protein ϕ_o from sea cucumber. *In*: JAMIESON, B. G. M., AUSIO, J., & JUSTINE, J.-L. (eds), Advances in Spermatozoal Phylogeny and Taxonomy. *Mém. Mus. natn. Hist. nat.*, **166** : 491-500. Paris ISBN : 2-85653-225-X.

Source : MNHN, Paris

Eukaryotic chromatin is a macromolecular nucleoprotein assembly essentially composed of DNA and basic proteins. Histones, which are relatively conserved through evolution, are the genuine protein components in chromatin of non-proliferating cells and are involved in the organization of the chromatin fibre into various structural hierarchies (for full review see [42]). In stark contrast, DNA in male germ cell lineages appears to be bound by widely diverse basic proteins as regards chemical composition and number, giving rise to a seemingly striking variety of protein molecules along the different zoological groups [32], spanning from those species which retain histones that are close to the somatic types, to those in which they are fully displaced by more basic proteins like nucleoprotamines [7]. The chemical data reported comprise a variety of vertebrate species, from fishes [9, 17] to mammals [25]. It is apparent that an enhanced basicity of the sperm proteins favours a tighter packaging of DNA. This requirement in spermatozoa seems obvious as a means of protecting the genomic complement. Likewise, it may also be an evolutionary adaptation of sperm to endure long-term storage and transport in the absence of DNA repair mechanisms. However, the functional significance of basic protein diversity in sperm and its effect at the molecular level are still ill-defined. DNA packing may not be the exclusive role. The actual existence of germ-line variants and sperm-specific protein types argues for more discriminating assignments such as fine-structural transitions of chromatin related to modulation of gene activity and its final quiescence during spermiogenesis [14].

The variability of sperm nuclear proteins is of unknown origin. A hypothetical evolution of some of these proteins from histone H1 has been put forward on the basis of the protein composition of the sperm of some bivalve molluscs [4]. Histone H1 and its many subtypes constitute the most heterogeneous of the histone classes [41]. Its largest evolutionary sequence-variation appears confined to both the N-terminal and C-terminal extensions of the molecules whereas the hydrophobic central globular core remains fairly well conserved [13]. This asymmetric organization has led to the suggestion that the carboxyl-terminus is involved in the higher order compaction of chromatin [1]. Models to test that assumption are provided by marine invertebrates, particularly echinoderms and molluscs. These organisms deserve particular mention since their somatic histones apparently coexist with both sperm-specific variants and protamine-like molecules, different from fish or mammalian protamines [3, 32, 43]. These protein molecules mostly fit into classes 3 and 4 of BLOCH's cytochemical categorization of nucleoproteins in mature sperm cells [7]. These two types have been ulteriorly combined into a rather heterogeneous group of intermediary sperm-specific types, plausibly representing transitions from histones to protamine-like molecules [32].

Although the prevalent cellular histones are encoded by a highly reiterated multigene family whose expression is tightly coupled to DNA replication, histone-variant genes tend to be present in single or few dispersed copies not subjected to cell cycle regulation [11, 23]. Already classification schemes based on regulatory correlations have been devised [43]. Nonetheless, very little is known about the organization of tissue-specific, variant-histone genes, along with the evolutionary origin of nucleoprotamine and protamine-like genes [30].

It is important to address these questions and obtain new evidence concerning histone to protamine transitions and the genes that encode them, aiming to understand at the molecular level their differential function and its influence on the structural organization of sperm chromatin. Our work has been involved in the analysis of chromatin from the germinal tissue of the echinoderm *Holothuria tubulosa*. During sperm maturation there is no bulk replacement of the histone complement, transitions being restricted to the addition of a sperm-specific, arginine-rich H1 variant [31] and the presence in ripe sperm of a small basic protein termed ϕ_o [40]. The latter has an amino acid composition reminiscent of the carboxy-terminal region of sea urchin H1-S, provided that Arg is considered equivalent to Lys [2]. Incorporation of protein ϕ_o into chromatin occurs in the terminal stages of spermiogenesis [10], representing about 4% of the histone moiety of the mature spermatozoa. Nucleosome organization remains invariable throughout sea cucumber spermatogenesis with a constant DNA linker length of 227 bp [15] consistent with sea urchin

Source : MNHN, Paris

nucleosomal repeats, which exhibit the longest lengths ever measured. The isolation and sequence determination of a cDNA for *H. tubulosa* protein ϕ_o have been previously reported [33]. In the present paper we describe the molecular cloning and characterization of the gene encoding this protein specific to the sea cucumber sperm chromatin. This is the first gene coding for a histone-to-protamine transition protein to be identified.

MATERIAL AND METHODS

Living organisms. Male specimens of the sea cucumber *Holothuria tubulosa* were collected periodically off the catalonian shore during the breeding season, moved live to the laboratory in cold seawater and held in 8°C seawater until used. Excision of gonads and sperm collection were performed as detailed elsewhere [36].

Isolation and purification of genomic DNA. High molecular weight genomic DNA was extracted from fresh sperm suspensions essentially as described [15]. Briefly, suspensions were treated with proteinase K (50 µg/ml) overnight at 37°C. After incubation, samples were deproteinized by successive phenol and chloroform extractions and the aqueous phases precipitated with ethanol. The DNA was further purified by Cesium chloride banding and subsequent dialysis.

Construction and screening of a sea cucumber genomic library. For construction of the *H. tubulosa* genomal library, purified sperm DNA was subjected to a partial digestion with *Mbo*I to generate fragments with *Bam*HI-compatible overhangs and subsequently size-fractionated by sucrose gradient centrifugation. DNA fragments in the size range 12-20 kb were pooled and ligated to dephosphorylated lambda-based Charon 35 (λCh35) replacement vector [26] linearized with *Bam*HI. Ligation reactions were carried out at a vector to insert molar ratio of 2:1. Recombinant phages were encapsidated and used to transform *E. coli* 555 *recA*$^-$ cells yielding a titre of $3x10^5$ plaque-forming-units (pfu) per µg of ligated DNA. The genomal library was screened by *in situ* plaque hybridization [5]. Plaques at a density of 10^4 pfu were replicated onto nitrocellulose membranes and screened with a 441 bp long *H. tubulosa* ϕ_o-cDNA clone [33] labelled by random-priming with the Klenow enzyme [21].

Positive plaques were purified by plating at decreasing densities and the isolated phages were grown by cascade infection and banded onto ethidium bromide-containing CsCl gradients. The resulting DNA was purified by phenol and chloroform extractions and used for further analysis. All recombinant DNA manipulations were carried out by standard procedures [37] and conducted in accordance with established guidelines for recombinant DNA research.

Restriction analysis and Southern transfers. DNA from positive recombinant clones was digested with selected endonucleases and pairwise combinations thereof. Where required, restriction fragments were electrophoresed on agarose gels, transferred to nylon membranes by alkaline blotting [34] after partial depurination and screened with six different regions of the ϕ_o-cDNA cloned insert as probes.

ϕ_o gene number. To assess the copy number of the ϕ_o gene in the haploid sperm genome of *H. tubulosa*, genomic DNA was digested, independently or in combination, with various endonucleases not cleaving inside the ϕ_o-cDNA sequence. Restriction fragments were subsequently electrophoresed on 0.5% agarose gels, blotted onto a nylon membrane, and hybridized to labelled probes prepared from the ϕ_o-cDNA clone. The ϕ_o gene number was derived by comparison of the number and intensity of the autoradiographic signals measured by densitometric analysis, with those from graded amounts of the cloned ϕ_o-cDNA equivalent to integer-copies per haploid genome of the cDNA sequence. Standards of cDNA were electrophoresed in parallel, supplemented with a mass excess of sheared calf thymus DNA to compensate for the amount of restricted sea cucumber sperm DNA loaded on each gel slot.

Plasmid subcloning and nucleotide sequence analysis. Genomic DNA restriction fragments of appropriate size identified by hybridization analysis as containing ϕ_o-cDNA sequence tracts were excised off the gel, purified further on low-melt agarose, and ligated into the phagemid vector Bluescript $^+$SK. Chimeric recombinants were used to transform competent *E. coli* XL1-blue *recA*$^-$ cells and were selected as AmpRTcR:Lac$^-$ phenotypes. DNA inserts from the recombinant plasmids were sequenced by the dideoxy chain-termination procedure [38] using the Sequenase system with forward and reverse primers for both orientations. Computer analysis was performed using the MicroGenie sequence analysis software (Beckman, USA).

RESULTS AND DISCUSSION

Spermatogenesis in the sea cucumber *H. tubulosa* is a rather simple process. Chromatin from ripe spermatozoa retains the five somatic-type histones in normal relative amounts accompanied by the highly basic protein ϕ_o (average M_r = 8640), structurally related to histone H1 [2]. We have previously reported the characterization of a clone carrying a full length ϕ_o transcript, isolated from a cDNA expression library made from the poly (A^+) fraction of total RNA extracted from immature gonadal tissue and screened with polyclonal anti-ϕ_o antibodies [33]. The 441 bp cloned cDNA encompassed a continuous open reading frame for a basic polypeptide of 77 residues whose sequence conformed to the partial amino acid sequence of ϕ_o previously established. Likewise, poly (A^+) selected RNA yielded a product electrophoretically

Source : MNHN, Paris

comigrating with protein ϕ_O upon *in vitro* translation in wheat germ, cell-free extracts, whereas Northern blot analysis detected only a 0.6 kb ϕ_O-mRNA transcript homologous to the cDNA probe.

Isolation of the sea cucumber ϕ_O gene

The genomal library of *H. tubulosa* sperm DNA cloned into the λCh35 vector was screened by hybridization with the ϕ_O-cDNA cloned insert. The screening of about 250 000 plaques yielded four positive clones with inserts of ~16.5, 14.4, 13 and 14.5 kb, named λHt7, λHt2, λHt1 and λHt8, respectively. The four recombinants were next subjected to endonuclease restriction and Southern blot analysis using six cDNA-derived probes encompassing specific regions of the ϕ_O-cDNA clone: (i) the two asymmetric segments resulting from the cleavage along the *Eco*RI site internal to the ϕ_O-cDNA insert, namely, the 248 bp leader (*5'* probe) and the 193 bp trailer (*3'* probe) fragments, encoding the first 69 and last 8 amino acids of protein ϕ_O, respectively; (ii) the 81 bp long *Eco*RI-*Pst*I fragment comprising the 5'-flanking region and the 42 bp sequence coding for the 14 amino acids inclusive of the initial methionine residue heading the N-terminus (*P* probe); (iii) the *Dde*I-*Eco*RI fragment of 81 bp representing amino acids 43 to 69 (*D* probe); (iv) the two segments of 113 bp and 80 bp in length (*X1* and *X2* probes) resulting from the nibbling of the *3'*-probe at the single *Xba*I site. Probe *X1* contained the sequence for the last 8 amino acids of the C-terminus plus 88 bp of the adjacent downstream extension whereas probe *X2* consisted of the final 80 bp of the 3'-noncoding region of the ϕ_O-cDNA clone. Those restriction fragments shown to carry ϕ_O sequences were subsequently subcloned for further DNA sequence analysis.

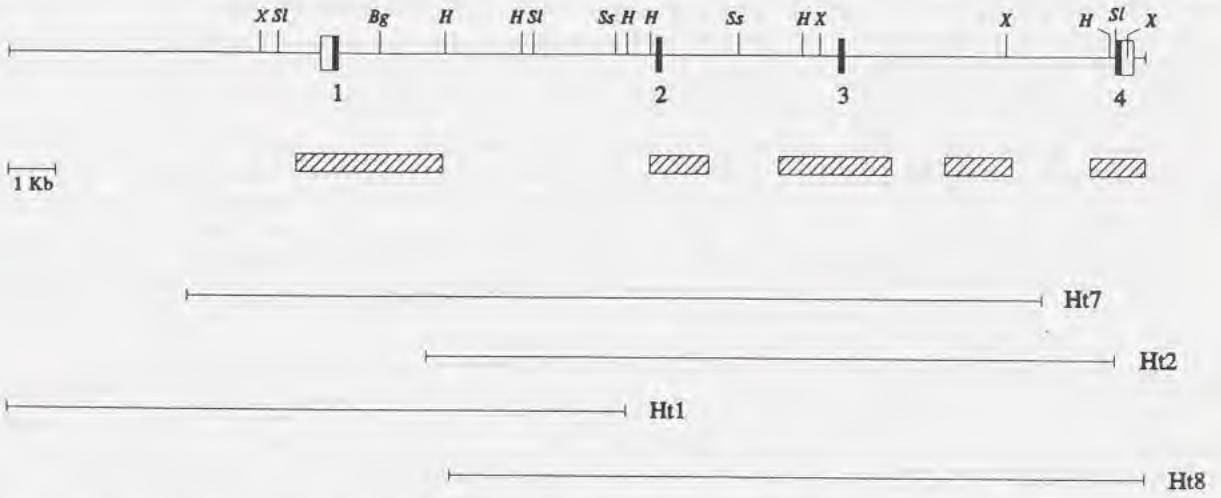

FIG. 1. — Isolation and characterization of the *Holothuria tubulosa* ϕ_O gene. The restriction endonuclease map and organization of the ϕ_O gene are shown. The four positive isolates picked out from the sperm DNA library of 10-20 kb *Mbo*I partials cloned into the *Bam*HI replacement vector λCh35, were digested with several endonucleases and combinations thereof. Restriction fragments carrying ϕ_O-cDNA sequences were identified by hybridization. Positively reacting fragments were further purified, subcloned into the phagemid Bluescript $^+$SK and sequenced by the dideoxy chain-termination procedure of Sanger. Filled boxes indicate the relative positions of exons encoding ϕ_O sequences consecutively numbered 1 to 4, and open boxes the 5' and 3' flanking regions homologous to the noncoding extensions of the cloned ϕ_O-cDNA. The hatched rectangular boxes highlight those regions of the gene that were completely sequenced either on both DNA strands or repeatedly in one direction. *B*, *D*, *E*, *H*, *P*, *Sl*, *Ss*, and *X* denote *Bgl*I, *Dde*I, *Eco*RI, *Hin*dIII, *Pst*I, *Sal*I, *Sst*I, and *Xba*I restriction sites, respectively. The thin lines depict the positions of the four strongly hybridizing genomic clones (λHt 1 - 8) used to map the ϕ_O gene.

Source : MNHN, Paris

The results of the restriction mapping are shown in Fig. 1 and can be summarized as follows. The entire ϕ_O gene appeared split into four distinct exon sequences scattered along the lengths of the clones. The latter displayed partial overlaps differing in extension. λHt7 harboured the 5'-proximal exons 1, 2 and 3. The central exons 2 and 3 were also present in λHt2 and λHt8. In addition, the former contained the first 79 nucleotides of the fourth exon whereas the latter spanned its entire coding sequence extending 831 nucleotides beyond the stop codon. The shortest clone λHt1 carried only exon 1 centrally positioned within the genomic DNA insert.

On the basis of these results it was feasible to correlate the four recombinants and to conclude that the coding sequence of the sea cucumber ϕ_O gene is interrupted by three long intervening sequences which amount to 16.2 kb in total length (see Fig. 1). These unusually large introns combine to make the ϕ_O gene the longest for a sperm-specific protein (*ca.* 17.7 kb) so far reported. The overall organization of this sea cucumber gene appears to significantly diverge from that of the intron-less histone genes [27] and also from the arrangement of mammalian, single-intron protamine genes [30].

Sequence analysis of the ϕ_O gene

The nucleotide sequence of the sea cucumber ϕ_O gene is shown in Fig. 2. The coding sequence is discontinuous and encompasses an open reading frame for a basic protein of 77 residues, interrupted by three introns involving canonical splice junctions [24], specifically those assigned to invertebrates [22]. The first and second introns of 6.8 and 4 kb in respective length, are inserted within codons 9 and 15, respectively. The third intron is 5.4 kb long and is positioned contiguous to codon 41. The complete coding sequence of the ϕ_O gene is identical to that of the cDNA clone previously reported [33] but for six nucleotide substitutions (97.9% homology). Five changes are conservative since they correspond to third-base degenerates of the most common synonym triplets, involving C for T and A for G conventional exchanges with no ensuing alteration of the assigned amino acid. The only relevant nucleotide substitution occurs at codon 61 and involves a G for A replacement in the first base causing a change of amino acid assignment. The overall level of sequence conservation observed argues for a stable organization of this gene.

The deduced primary structure of the encoded protein corresponds exactly with the ϕ_O sequence specified by the cDNA clone, exclusive of the noted single alteration at codon 61 (98.7% homology). The cDNA sequence contains the triplet GCC for alanine in this position while the ACC counterpart in the gene codes for threonine. Most likely this difference arises from the DNA polymorphism detected in echinoderms [12] which is reflected in the well-documented intraspecific microheterogeneity found in a substantial variety of sperm variant proteins from marine invertebrates [28] as well as in the protamines of trout [18].

The close sequence homologies between the ϕ_O-cDNA and the cloned gene are endorsed by the identity of the deduced protein sequences which, in addition, reproduce wholly unaltered the partial amino acid sequence of protein ϕ_O previously established. The coincident similarities observed sustain the conclusion that, in actuality, the cloned gene encodes the sperm-specific protein ϕ_O in *H. tubulosa.*

Comparison of the nucleotide sequences in the noncoding regions of the ϕ_O gene with those of replication-dependent histone genes reveals that the former lacks the conserved motifs defining the S-phase regulated histone gene structure such as the downstream hairpin loop sequence at its 3' proximal purine-rich tract required for 3' processing of the histone transcripts [6]. Instead, the leader and trailer regions surrounding the ϕ_O gene combine several structural elements found in both replication-independent histone variant as well as protamine genes (see Fig. 2).

The region upstream of the initiation codon contains an atypical TATA motif identical to the TTCAAA box identified in the cell-cycle independent $H2A_F$ histone gene that codes for an extreme H2A variant in chicken, whose transcript is polyadenylated [16]. Significantly, both

Source : MNHN, Paris

elements are found similarly positioned 144 nucleotides upstream from the initiator triplet. There is another potential TATA motif with perfect homology to the non-canonical TTAAAT element present in both the chicken and duck H5 genes [19, 35]. This sequence starts at position -205, 65 and 28 nucleotides further upstream than the mentioned homologues, respectively. Another general feature required for promotion of transcription by RNA polymerase II is a CAAT sequence often located between 70 to 90 bp upstream of TATA sites [8]. In this regard, the leader region of the ϕ_O gene displays a potential CAAT motif (-221 to -218) located 73 nucleotides upstream relative to the $H2A_F$-like TTCAAA box. Another motif shared with leader regions in protamine genes is the TGACGTCA sequence found far upstream in the ϕ_O gene (-489 to -482). This *cis*-acting element, usually referred to as a cAMP regulatory element (CRE), is strictly conserved in all protamine genes and it is considered essential for the biological activity of cAMP-regulated enhancers [30]. Since spermatogenesis in echinoderms is known to be under hormone control probably involving cAMP [39], such a regulatory signal might well represent a link between hormonal signals and the expression of sperm-specific genes such that of protein ϕ_O.

The downstream extension of the ϕ_O coding sequence is devoid of the highly conserved structural features of the replication-dependent histone genes required for the 3' end formation of histone transcripts. Instead, similarities with the equivalent regions in protamine genes are encountered. First, three potential polyadenylation signals are present, starting at positions 306, 392 and 396 respectively, 3' to the TAA stop codon. The last two elements consist of the heptamer AAATAAA which appears repeated with a trinucleotide overlap. This heptameric sequence motif bears a perfect homology with the conserved polyadenylation signal found in the protamine genes from salmon and trout [29]. Nonetheless, the significance of the close similarities encountered between the organization of the ϕ_O gene and that of genes coding for extreme histone variants or even for protamines remains to be unambiguously defined.

Genomic content of the ϕ_o gene

The copy number of the ϕ_O gene was determined by Southern blot hybridization analysis of sperm DNA restriction digests with the 81 bp long *Eco*RI-*Pst*I fragment (probe *P*) of the ϕ_O-cDNA clone comprising the 5'-flanking extension and the initial 42 bp of the coding sequence, labelled by random priming. A set of endonucleases lacking cleavage sites within the cDNA sequence was selected for the single and double enzyme digestions. DNA restriction fragments were electrophoretically resolved in conjunction with varying amounts of the cloned ϕ_O-cDNA insert, diluted with a mass excess of sheared heterologous DNA to make up for those of restricted sperm DNA loaded on the gel lanes. The amounts of the cDNA standards, equivalent to one and four copies of the respective sequence per haploid genome, were inferred from the DNA content of the haploid genome (C-value = 3 x 10^9 bp) of *H. tubulosa* sperm previously determined [36].

Hybridization patterns from both single and combined enzyme restrictions yielded in every case only one size class of DNA fragment positively hybridizing with the cDNA probe (Fig. 3).

FIG. 2. — DNA sequence of the sea cucumber ϕ_O gene and flanking regions. The 5'→3' nucleotide sequence of the non-transcribed strand (i.e. mRNA-like) is given along with the amino acid sequence derived from the ϕ_O coding region, shown above the nucleotide sequence. The abbreviation *ini* and the asterisk mark the respective positions of the initiator and the stop codons. The noncoding leader and trailer extensions are numbered with negative and positive numerals begininng at nucleotides 5' adjacent to the initiation codon and 3' proximal to the stop triplet, respectively. Coding triplets are denoted with numbers in *italics*. The slash symbols mark the normal donor splice site junctions. Positions of the putative CRE, CAAT and TATA elements as well as the poly(A) addition signal discussed in the text, are doubly underscored. Most of the extensive intron sequences have been removed for clarity.

Source : MNHN, Paris

Exon 1

```
                                -700                                                  -650
GACTGCTCACGAGTGATAGCGCCCAAACAAGCGTCCAAGAGGCCGAACAGACGCTAAGGCTCGCTACGGCGCGTTAGCAT
                                                        -600
GTACAAAATTGCCTCGGCGTTTTCACTAGTATACGCGTTTCTACGGCGATGGAATCGACTTGAGTAAGCTGACCTACATC
                    -550                                                  -500
ACGTGACCTTCCATGATGGCTTCACAACAACACTGTGGGATGCGCCTTCCTGTTATATAGTTCAGTGGAGCCGATGCTTA
                                                -450
TGACGTCATAATTTGGTGTAGAAGTCTAGTCCACTGCATATTTCCAAACGTGAAGAAACGGTTTGAGACCGAATCAAATG
        -400                                                  -350
CATTTTTCTTACCAGCTTACCGCGCTCTGGAATACGATGAGTATGTATGCAATCCTCAGTTCAAAGCAAAGTGGACGCAC
                                -300                                                  -250
TACGTTTGGACACTGTACCTAGCCTACATTATTCATGTTCTTGTTCCCTTTATGCACGTAAGGACGCAACCCGATGCGAC
                                                        -200
GTTTCTGAAACGGGCACTTAGACGCGCGCAATTTGTAATCACGTTTAAATCATAGTATTTGGTGCGCGTAGCTATAGGTG
                    -150                                                  -100
CGTTTATGCGCCTCACTTTGTAAAATTCAAAAAAATAACAAATATTGTTTTCAGTTTTTAAAGAACCGTTACTTGATCGC
                                                -50
TTACAGCCGAGCAACTAAGACGTTGGTCCTACGCACTGCCCAGTTTTGATTCCCCCTTGTGTCGGAAATTCCAACTACAA
        -1                          5
TCAATAATC ATG GTA GCC AGA CGA CAA ACA AAG AAA G/GTAAATAAAGGGAACAATATATGCAAGGCGTT
          ini Val Ala Arg Arg Gln Thr Lys Lys A
```

Exon 2

```
                                        10                  15
TTTTATTTCTTCTTTCTCACTTCACAG CT AGG AAG CCT GCA GCC AG/GTGAGTGAATACAATTTAAATTTTAT
                                la Arg Lys Pro Ala Ala Ar
```

Exon 3

```
                                                                20                  25
TAACTCAAGACTTTAAATGCTTTGTCCTTCCTCCCCAG G AGA CGC AGC GCA GCC AAA CGC GCA GCC CCA
                                        g Arg Arg Ser Ala Ala Lys Arg Ala Ala Pro

                    30                  35                  40
GCC GCG AAG AAG GCT GCG AGT CGC CGT CGC CCA AAG AGT GCT AAG AAG/GTAGGTAATAAGATGT
Ala Ala Lys Lys Ala Ala Ser Arg Arg Arg Pro Lys Ser Ala Lys Lys
```

Exon 4

```
                                                            45                  50
AATTAAAAAAAGTCCTGACAATATATTTTTCTCTTTTCAG GCT AAG CCC GCA GCA AGG AGG CGC AGC AGC
                                         Ala Lys Pro Ala Ala Arg Arg Arg Ser Ser

                55                  60                  65                  70
GTC AAA CCT AAA GCA GCA AAA GCA GCC ACC CAA GTC CGT CGC AGG AGC CGA CGA ATT CGC
Val Lys Pro Lys Ala Ala Lys Ala Ala Thr Gln Val Arg Arg Arg Ser Arg Arg Ile Arg

                75                  +1                                          +50
CGT GCG TCC GTG TCA AAG TAA TTCAATGGAAGACTGATCATTAAATCGTAACCCCTTCGAAAGATTAAACTTA
Arg Ala Ser Val Ser Lys  *
                                                    +100
TCAAATTTCATTTTGTAGAACTGTCCAAATTTTCTAGAATATTGCAGAACTGAACATTTAAAACACATCCAAATTCGTAA
                +150                                                  +200
GCGAACAAGCAAGCAACGATGACCTACAATTTACAGTCGTTTCTTATTATTTCAAGTTTGCCTTTATTCAGTTTCAGTTT
                                        +250
CAGTTTATTTACTCTTTAATACCTCCTCGGAGGTGTCAGAGTCAAAACATACAATTAGATACACAGAAATATACAAAAAG
    +300                                                  +350
CAGCAAGATCAACAATAAACAAAAACAAAAAACAAAAATCATGCAAGCAAATCAGTACAATCAAAAAACTAACTTCAACC
                            +400                                                  +450
TAGTCACCAGACCCAATTGAAATAAATAAATCCAGAACAGAAATTGGATAGGCATGCATGAACTAGACGTAACACAAACA
                                                    +500
TACGCACAACACAACGAGCAACCTACGTATGCACTAATGCCTAGCTATACACACTACATCATCAAATCAACTACGGCACT
                +550                                                  +600
CATTAACGATGATATACACTACAGAAGTGGCCAGGTTTCTTTGCACCTCCTTATTTCTAGACGTTAAAAGCTGGGCCATT
                                        +650
TTTAAAGTATTTGGACCCACCCTATAATAATGCTTCAAATAGTATTTCCGATCTGAGTTGAAAGCCTTACATTGAAAAAG
    +700                                                  +750
ATAATGAAATTACATCTCCTATCAGCCCACTAGTACAAAGTTCACAAAGTCTATCATTAAAGTCAATATTAAGAAATCGT
```

Source : MNHN, Paris

In turn, each singly-reacting fragment appeared to hybridize with the probe to a similar extent as revealed by the intensity of the autoradiographic signals estimated from densitometer tracings. Comparison of the level of hybridization of the genomic fragments with the quantified signal intensities of the cDNA standards yielded an average value of 0.92 copies of the ϕ_O gene per haploid genome. These results indicate that the *H. tubulosa* sperm ϕ_O protein is probably specified by a single copy gene.

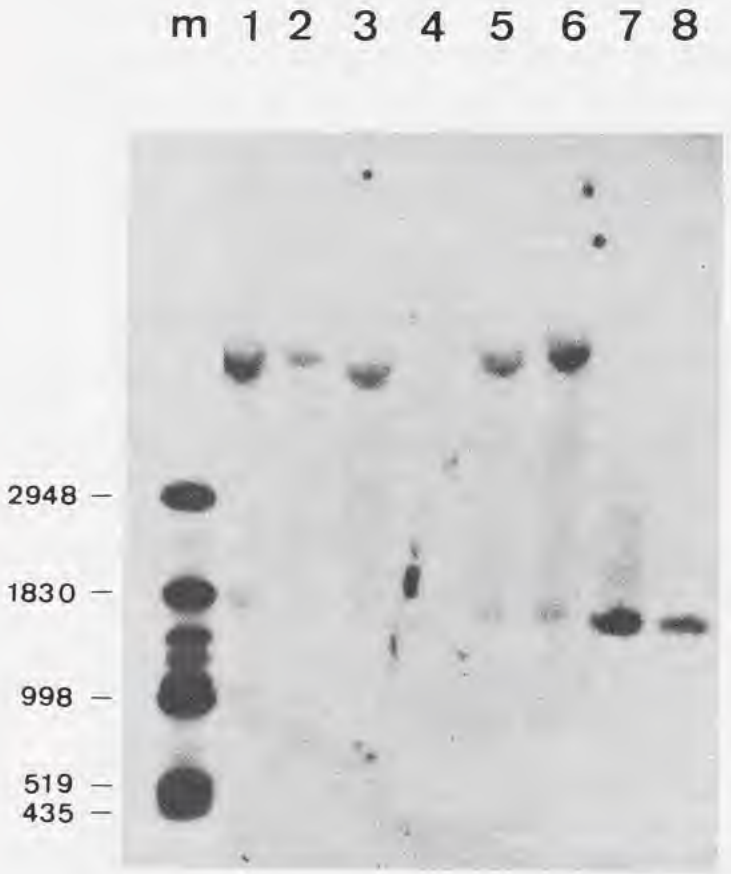

FIG. 3. — *H. tubulosa* ϕ_O gene copy number. Autoradiogram of Southern blots of sea cucumber sperm DNA restriction digests hybridized with *P*-probe of the cDNA (see text for details). Sperm DNA samples (20 µg) were digested with: lanes 1 to 6 *Bam*HI; *Hin*dIII; *Kpn*I; *Bam*HI + *Hin*dIII; *Bam*HI + *Kpn*I; *Hin*dIII + *Kpn*I, respectively. Graded amounts of the ϕ_O-cDNA cloned insert equivalent to 4 (lane 7) and 1 copies (lane 8) per haploid genome, supplemented with a mass excess of sheared calf thymus DNA were co-electrophoresed as hybridization standards. Sizes of restriction fragments from the ϕ_O-cDNA clone run in parallel as migration markers, are given in bp (lane m).

The structural arrangement and content of the ϕ_O gene closely coincide with the common features of a sizable number of post-meiotically expressed genes, typified by those encoding most histone-to-protamine transition proteins as well as protamine genes [20, 23]. Besides being expressed in a replication-uncoupled manner, these genes usually generate polyadenylated transcripts and most of them, although not all, are present as single copies containing coding regions often interrupted by intervening sequences. The overall organization of these genes becomes clearly divergent from that of the somatic histone genes. The functional implications, if any, of this divergence in gene arrangement remains unexplained, although potential correlations with chromatin transitions related to modulation and final arrest of gene activity during the spermiogenic process should be taken into account. Further studies are underway to characterize new genes encoding known sperm-specific protein variants.

Source : MNHN, Paris

ACKNOWLEDGEMENTS

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Source : MNHN, Paris

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Source : MNHN, Paris

Nuclear Basic Proteins from the Sperm of Tunicates, Cephalochordates, Agnathans and Fish

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ABSTRACT

In this chapter we try to review and arrange the studies on sperm nuclear basic proteins (SNBPs) carried out in several groups of deuterostomes (tunicates, cephalochordates, agnathans, chondrichthyans and osteichthyans). Four general points arise: 1. There are two main types of SNBPs: a. proteins similar to histones but with enhanced basicity, named here "PL" (protamine-like) and b. very specialized proteins, named here "P"; 2. The"PL" proteins have appeared independently several times during deuterostome evolution; 3. In some cases, "P" proteins may have arisen from "PL" proteins, but other origins can not be ruled out for a particular P protein; 4. The classical evolutionary point of view about the appearance of protamines (histones → intermediate proteins → protamines) is re-interpreted as histones → PL proteins → P proteins in this paper. This transition seems to have repeatedly occurred during the evolution of different groups of deuterostomes. Nevertheless, it should not be interpreted as a continuous evolutionary line of the sperm proteins of the whole deuterostome line. In other words, there does not exist an apparent continuous evolutionary line relating the SNBPs of echinoderms with the bony fish and tetrapod protamines.

RÉSUMÉ

Les protéines nucléaires basiques des spermatozoïdes chez les Tuniciers, Céphalocordés, Agnathes et Poissons

Nous essayons dans ce chapitre de faire une synthèse des études concernant les protéines nucléaires basiques des spermatozoïdes (PNBS) qui ont été effectuées sur plusieurs groupes de Deutérostomiens (Tuniciers, Céphalocordés, Agnathes, Chondrichthyens et Ostéichthyens). Quatre points généraux émergent: 1. Il existe deux types principaux de PNBS: a. des protéines similaires aux histones mais avec une basicité augmentée, nommée ici "PL" (proches des protamines), et b. des protéines très spécialisées, nommées ici "P". 2. Les protéines "PL" sont apparues indépendamment plusieurs fois pendant l'évolution des Deutérostomiens. 3. Dans certains cas, les protéines "P" peuvent être apparues à partir des protéines "PL", mais d'autres origines ne sont pas à exclure dans le cas de certaines protéines P. 4. Le point de vue évolutif classique concernant l'apparition des protamines (histones → protéines intermédiaires → protamines) est ré-interprété dans cet article comme: histones → protéines PL → protéines P. Cette transition semble s'être produite de manière répétée pendant l'évolution des différents groupes de Deutérostomiens. Néanmoins, elle ne doit pas être interprétée comme une ligne d'évolution continue des protéines des spermatozoïdes dans l'ensemble de la lignée des Deutérostomiens. En d'autre termes, il n'existe pas de ligne d'évolution continue reliant les PNBS des Échinodermes aux protamines des Poissons osseux et des Tétrapodes.

CHIVA, M., SAPERAS, N., CÀCERES, C. & AUSIO, J., 1995. — Nuclear basic proteins from the sperm of tunicates, cephalochordates, agnathans and fish. *In*: JAMIESON, B. G. M., AUSIO, J., & JUSTINE, J.-L. (eds), Advances in Spermatozoal Phylogeny and Taxonomy. *Mém. Mus. natn. Hist. nat.*, **166** : 501-514. Paris ISBN : 2-85653-225-X.

Source : MNHN, Paris

In recent years, we have perfomed a series of studies on the SNBPs in deuterostomes intended to link information already available from echinoderms [44], and from several groups of fishes and tetrapods [reviewed in 22, 31-32]. The sperm nuclei of echinoderms contain somatic histones or specific histones with specialized N- and C-terminal domains. In contrast, the SNBPs from fish (also referred to as "typical protamines") are very short proteins (about 30 amino acid residues), consisting mainly of arginine and a few other types of amino acid residues [31]. Two hypotheses have been proposed for the evolution of SNBP in deuterostomes. One of them relates the SNBPs from echinoderms to fish protamines [44]. The second one proposes a foreign origin for the "typical protamine" of bony fish [21, 27]. However, there was a complete absence of information about the SNBPs of deuterostomes from intermediate taxonomic groups. In this chapter we arrange the information already available on the SNBPs of these groups, which may help to clarify some basic and general aspects of the evolution of these molecules. Yet there are several major difficulties in establishing the evolutionary link amongst different SNBPs. They stem from the remarkable variability of these proteins, the limited amount of information still available and the lack of a general consensus about the phylogenetic relationship among different groups of deuterostomes [12, 45].

RESULTS

SNBPs from Tunicates

In recent years, we have studied the SNBPs of several species from four different families of ascidiacean tunicates [9-10, 36]. The main conclusion from these studies is the general constancy in the SNBP pattern exhibited in each of them. Figure 1A shows the electrophoretic patterns of four species, each one belonging to a different family. In all cases, they consist of a major protein (PLasc) (asc = ascidiacean) which migrates close to the position corresponding to histone H4. Each one of the patterns shown in Fig. 1 is representative of all the studied species belonging to the same family, except in the case of the family Styelidae. Within this family, the two species studied of the genus *Styela (S. plicata* and *S. montereyensis)* display a slightly more complex SNBP electrophoretic pattern (Fig. 1B). *Styela* species contain, in addition to the PLasc protein, a significant amount of other proteins.

The PLasc proteins consist of about 150 amino acid residues, and are rich in arginine, lysine, and relatively rich in glycine (Table 1). The studies carried out to date [35, 36] indicate that PLasc exhibits the same tripartite structural organization which is characteristic of histone H1 [19]: An N- and a C-terminal tail flanking a central hydrophobic core (Fig. 1C). The N-terminal region has only two arginine residues (NH_2-R-R-). The trypsin-resistant core consists of 74 amino acid residues and has an amino acid composition (Table 1) and sequence [35] that ressemble those of the trypsin-resistant cores from both H1 histones [1] and some of the SNBPs from bivalves [3] and gastropod molluscs [15]. The C-terminal tail is very basic (Table 1) and its sequence does not display any similarity with the N- or C-terminal tails of the SNBPs from echinoderms ([35] and unpublished results).

In the case of the genus *Styela*, the slow migrating protein (PLsty.1 in Fig. 1B) contains

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NOTE:

In this article the PL proteins of small molecular weight resulting from post-translational cleavage of larger PL precursors have been generically referred to as P proteins. This nomenclature has been adopted to emphasize the structural similarity existing between these proteins and protamines as discussed in the preceding article (AUSIO, this volume).

ABBREVIATIONS:

aa = amino acids, AUT = Acetic Acid/ Urea/Triton Gel Electrophoresis, SDS = Sodium Dodecylsulfate, SNBPs = Sperm Nuclear Basic Proteins

Source : MNHN, Paris

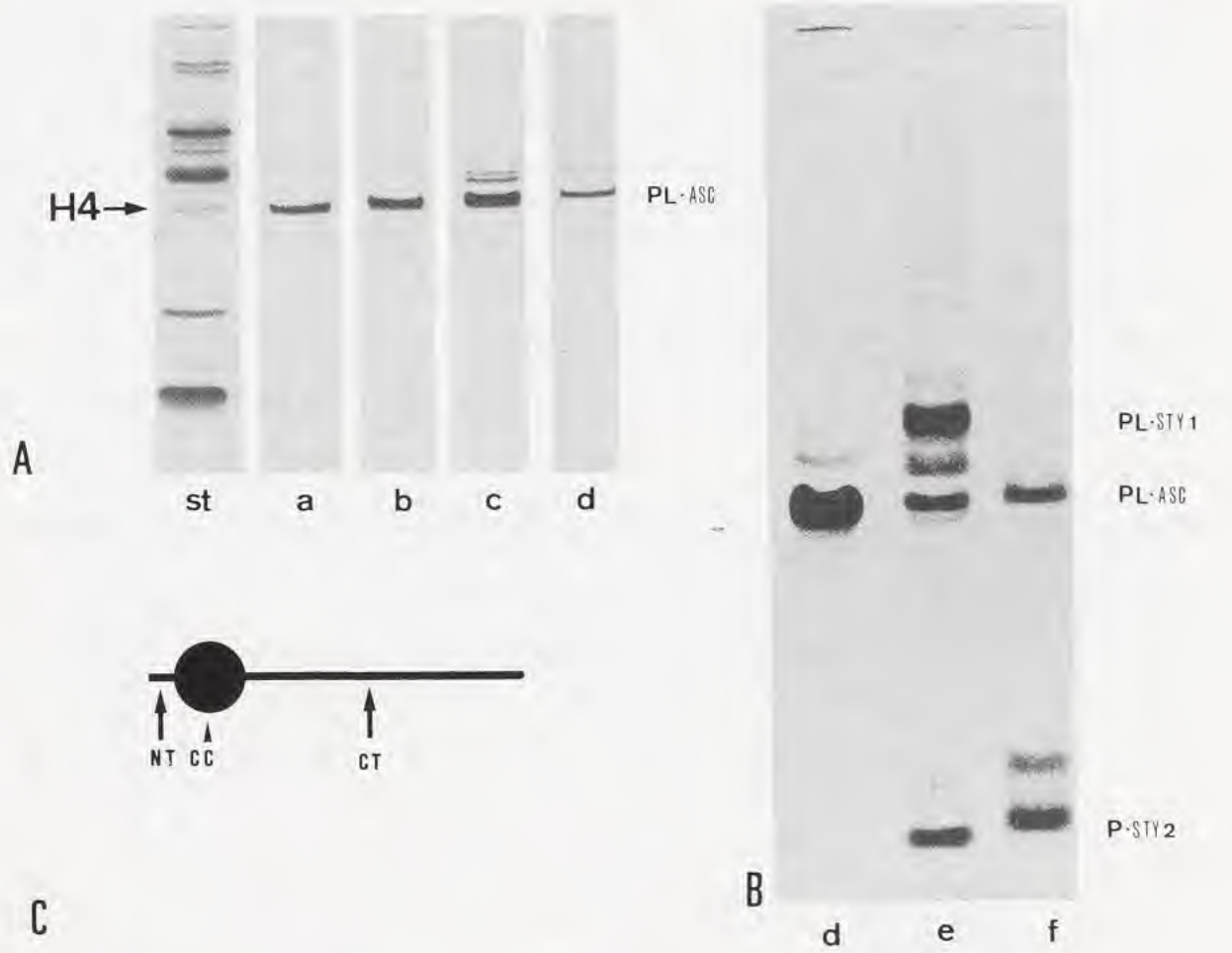


FIG. 1. — **A:** Electrophoretic pattern of SNBPs from four ascidiacean tunicate species. Each species belongs to a different family: a, *Ascidia callosa* (Ascidiidae); b, *Chelyosoma productum* (Corellidae); c, *Boltenia villosa* (Pyuridae); d, *Cnemidocarpa finmarkiensis* (Styelidae); st, proteins from the sperm of the ratfish *(Hydrolagus colliei)* used as a marker standard [10]. **B:** Comparative electrophoretic analysis of PLasc (lane d) and the SNBPs of two species from the genus *Styela*; e, *S. plicata*, f, *S. montereyensis*. The direction of electrophoresis is from top (+) to bottom (-). **C:** Schematic representation of the structural organization of the PLasc molecule; NT: amino-terminal domain (2 arginine residues); CC, trypsin-resistant hydrophobic core (~75 amino acid residues); CT, carboxy-terminal domain (70-75 amino acid residues).

also a trypsin-resistant peptide and an amino acid composition similar to that of PLasc (Table 1) [35, 36]. The protein Psty.2, of higher electrophoretic mobility, displays microheterogeneity and consists of three distinct forms that can be resolved by HPLC [36] and that exhibit an almost identical amino acid composition (Table 1). Table 1 also shows the enormous compositional resemblance that exists between the C-terminal tail of PLasc and protein Psty.2. This fact is of a special interest because it suggests a close relationship between these two molecules (see discussion).

In summary, the sperm nuclei of the ascidiacean tunicates consist of a protein (PLasc) which is different both from specific sperm histones of echinoderm and from the "typical protamines" of the bony fish. This protein appears in all families studied and may represent the

Source : MNHN, Paris

TABLE 1. — Amino acid composition (mol %) of the SNBPs from tunicates (a-g) and from the cephalochordate *Branchiostoma floridae* (h). As a comparison, the composition of a PL III protein (a SNBP from the bivalve mollusc *Crenomytilus grayanus* [29]) is shown in lane (i); a, protein PLsty.1 (PL of *Styela plicata*); b, PLasc (*S. plicata*); c, trypsin resistant core of PLasc (*S. plicata*); d, C-terminal domain of PLasc (*S. plicata*); e-g, each one of the three components of Psty.2 protein (*S. plicata*).

	a	b	c	d	e	f	g	h	i
Lys	14.8	14.3	12.5	9.1	15.8	16.9	17.4	24.7	25.4
His	1.0	1.1	2.1	-	-	-	-	-	-
Arg	33.8	32.5	11.5	58.2	50.8	49.5	50.4	25.3	26.5
Asx	5.1	4.6	8.9	-	0.2	-	1.2	-	0.9
Thr	2.8	1.2	1.2	1.8	2.0	3.1	3.1	-	3.4
Ser	5.3	4.6	8.1	-	1.4	1.4	1.1	16.5	15.6
Glx	2.6	2.2	1.4	3.6	0.3	0.7	-	-	0.9
Pro	2.5	1.4	3.1	-	-	-	-	5.6	5.1
Gly	12.0	13.2	8.1	20.0	21.5	20.8	18.7	6.1	6.5
Ala	8.1	7.1	10.2	3.6	4.5	3.4	3.3	21.7	12.9
1/2 Cys	-	0.7	2.5	-	-	-	-	-	-
Val	4.2	4.6	9.1	-	0.2	-	-	-	1.5
Met	-	1.4	3.3	-	1.3	0.7	0.7	-	-
Ile	1.5	2.4	2.3	-	-	-	-	-	0.4
Leu	2.7	4.5	6.8	3.6	1.9	2.5	3.3	-	0.6
Tyr	2.3	2.2	4.5	-	-	0.9	0.7	-	0.1
Phe	1.3	2.2	1.3	-	-	-	-	-	0.1

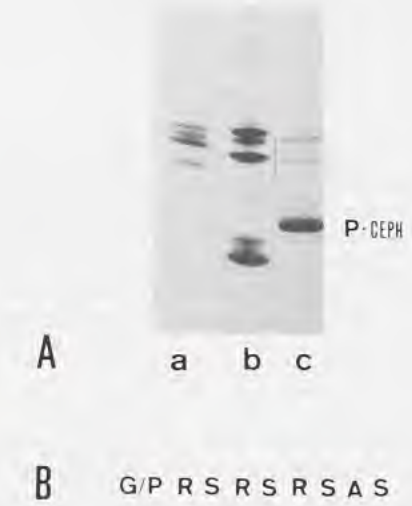


FIG. 2. — **A:** Electrophoretic pattern of *Branchiostoma floridae* SNBPs (lane c), shown in comparison with the SNBPs from the tunicate *Styela plicata* (lane b) and a chicken erythrocyte histones standard (lane a). **B:** First nine amino acid residues (N-terminus) of Pceph protein.

ancestral SNBP of ascidiacean tunicates. Nevertheless, in some particular groups, as in the genus *Styela,* some variations to this general protein pattern have occurred; namely, the appearance of a shorter and more basic molecule (Psty.2) in addition to molecules similar to PLasc (for example, PLsty.1).

Source : MNHN, Paris

SNBPs from Cephalochordates

The phylum Cephalochordata consists of two genera (*Branchiostoma* and *Epigonichtys*) [28]. The SNBPs from *Branchiostoma floridae* have partially been characterized [40]. Their electrophoretic pattern (shown in Fig. 2A) displays a main protein component (Pceph in Fig. 2A) which is accompanied by small quantities of residual histones [40]. Pceph has an electrophoretic mobility similar to that of tunicate protein Psty.2. Its size, estimated from the electrophoretic behaviour is of about 120 amino acid residues, and its compositional analysis (Table 1) is very simple. The protein consists of only six different types of amino acid residues, with arginine, lysine, alanine and serine being the most abundant. It is interesting to note, with regard to the amino acid composition and size, that Pceph is more similar to SNBPs from bivalve molluscs than to other deuterostome SNBPs (Table 1). The N-terminal sequence (shown in Fig. 2B) contains three alternating arginine-serine motives. Such an alternation of basic (R/K) and phosphorylatable residues (S/T) has also been observed in the SNBPs from several gastropod and bivalve molluscs [6, 13, 17], birds and mammals [30-31], but it is not present in the "typical protamines" from bony fish (Fig. 5B).

In summary, the protein Pceph shares some characteristics with typical protamines such as its low amino acid diversity, its basic composition and the presence of serine. Yet, Pceph and "typical" protamines have several distinctive features. First, in Pceph lysine and arginine are present in similar proportions (24.7% and 25.3% respectively) whereas protamines consist almost exclusively of arginine. Secondly, Pceph displays an alanine rich amino acid composition (21.7%) not found in protamines. Finally, Pceph has a much larger size than "typical protamines" and contains the repetitive motifs arg-ser which is absent in bony fish protamines.

TABLE 2. — Amino acid composition (mol %) of the sperm histones of the lamprey *Petromyzon marinus* (PM) in comparison to the somatic histones from calf thymus (CT) [26].

	H1		H2A		H2B		H3		H4	
	PM	CT	PM	CT	PM	CT	PM	CT	PM	CT
Lys	27.6	26.8	9.7	10.2	14.1	14.1	8.3	10.0	10.4	11.4
His	-	-	1.6	3.1	3.3	2.3	1.7	1.7	1.8	2.2
Arg	2.2	1.8	9.8	9.4	8.2	6.9	13.8	13.0	13.4	12.8
Asx	3.9	2.5	6.3	6.2	4.8	5.0	4.2	4.2	4.8	5.2
Thr	3.6	5.6	3.2	3.9	6.6	6.4	7.5	6.8	6.7	6.3
Ser	7.3	5.6	5.2	3.4	10.3	10.4	4.5	3.6	2.0	2.2
Glx	4.7	3.7	9.4	9.8	6.4	8.7	12.0	11.6	6.5	6.9
Pro	9.7	9.2	4.1	4.1	6.2	4.9	4.5	4.6	1.4	1.5
Gly	4.0	7.2	11.4	10.8	5.9	5.4	5.6	5.4	16.8	14.9
Ala	21.8	24.3	13.1	12.9	11.6	10.8	13.3	13.3	7.9	7.7
Cys	-	-	-	-	-	-	0.6	1.0	-	-
Val	6.8	5.4	7.2	6.3	6.0	7.5	4.2	4.4	8.1	8.2
Met	-	-	0.5	-	2.1	1.5	0.8	1.1	0.2	1.0
Ile	1.1	1.5	3.7	3.9	5.8	5.1	4.7	5.3	5.5	5.7
Leu	5.3	4.5	11.9	12.4	4.5	4.9	8.9	9.1	8.6	8.2
Tyr	0.8	0.9	2.3	2.2	2.8	4.0	2.2	2.2	3.6	3.8
Phe	1.1	0.9	0.9	0.9	1.6	1.6	3.3	3.1	2.1	2.1

Source : MNHN, Paris

SNBPs from Agnathans

The nuclear sperm proteins from Agnatha have only been studied in one species of lamprey, *Petromyzon marinus* [40]. Nuclei isolated from ripe sperm of *P. marinus* consist only of histones (Fig. 3A-C). According to the chromatographic and electrophoretic behaviour as well as to the compositional amino acid analyses (Table 2), the sperm histones of *P. marinus* do not exhibit any specific characteristics that distinguish them from the somatic type. This is surprising considering that agnathans have appeared relatively late in evolution and it contrasts with the strong tendency of tunicates and cephalochordates to have specialized proteins in their sperm nuclei. It is necessary to study the SNBPs of more agnathans in order to determine whether the absence of specialized nuclear sperm proteins is just a specific characteristic of *P. marinus* or represents the general trend in agnathans. Nevertheless, the absence of specific proteins and the presence of somatic-like histones in the sperm nucleus also occurs in some groups of bony fish (see below).

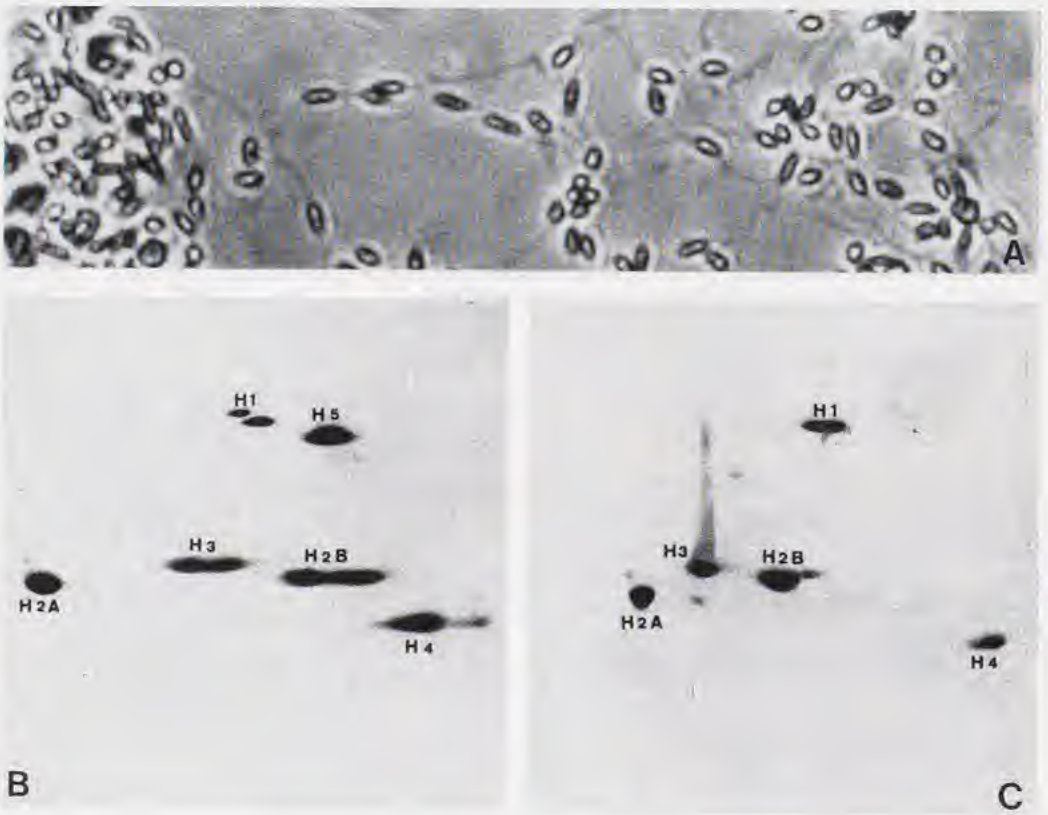


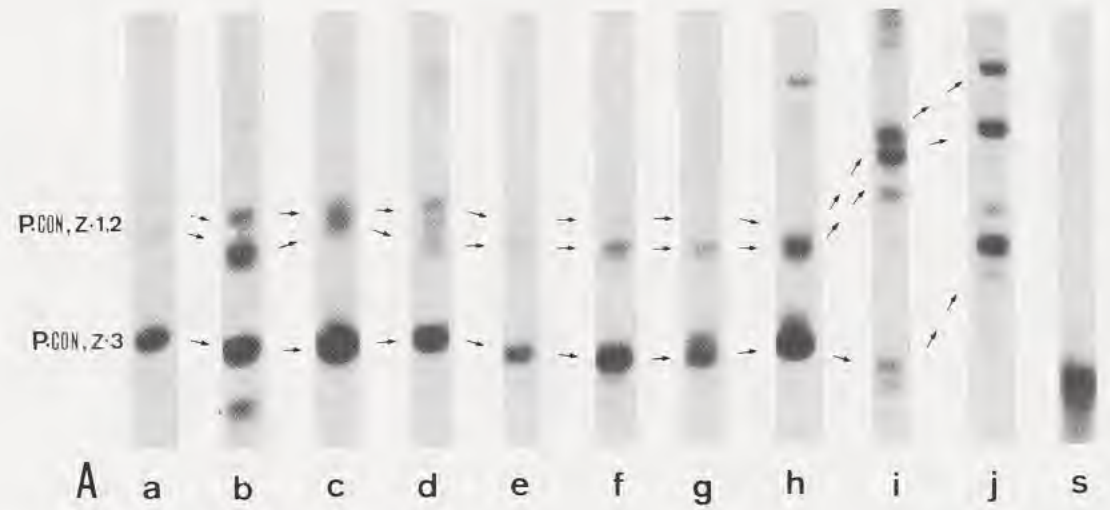
FIG. 3. — **A:** Light microscopy micrograph of ripe sperm used for the analysis of the nuclear sperm proteins of *Petromyzon marinus* (x 1 000). **B, C:** Two-dimensional electrophoretic protein patterns of SNBPs from *Petromyzon marinus* (3C), and histones from chicken erythrocyte (3B) used as standard. The direction of the electrophoresis was left (+) to right (-) (first dimension, AUT) and top (-) to bottom (+) (second dimension, SDS).

Source : MNHN, Paris

SNBPs from Chondrichthyes

The class Chondrichthyes consists of two lineages that diverged very early in its evolution: Holocephali and Elasmobranchii [28]. The Elasmobranchii diverged later giving raise to the Selachimorpha (sharks) and the Batidoidimorpha (skates). The electrophoretic patterns of the SNBPs from eight selachians, one batoidean and one holocephalan species are shown in Fig. 4. All contain several basic proteins in their sperm nuclei. One of these proteins (Pcon.Z3 in Fig. 4) can be directly extracted with dilute acids. However, the other major proteins (Pcon-Z1, Z2) require chemical reduction of the nuclei before they can be solubilized by acids. The need for reduction prior to protein extraction is a characteristic of cysteine-rich proteins owing to the formation of intermolecular S-S bridges in the ripe sperm nuclei. The SNBPs from the selachian *Scyliorhinus canicula* have been characterized previously [18] and they are representative of the SNBP pattern in all selachian species (see the similarity among selachian SNBPs in Fig. 4A). The electrophoretic pattern of the SNBPs of one batoidean species *(Raja rhina)* is shown in Fig. 4A. Unfortunately, there is no information available on the amino acid composition of batoidean SNBPs. The holocephalan *Hydrolagus colliei* contains several minor proteins and three major proteins [37] (see also Fig. 4A). The amino acid compositions of the main *H. colliei* SNBPs are shown in Table 3 in comparison with the corresponding proteins form *S. canicula* .

In Fig. 4B we compare the N-terminal sequence of Pcon-Z3 from *H. colliei* [37] with the



B

(a) ARRRHSMKKKRKSVRRRKTRKNTRKRKNSLGR

(b) ARSRSRRSYGRGRRRGGRRRRRRRRRRRRGGR

FIG. 4. — **A:** Electrophoretic pattern of the SNBPs of eight selachian species (a-h), one batoidean (i) and one chimaeriform (j). a, *Galeus melastomus*; b, *Scyliorhinus canicula* (a, b, family Scyliorhinidae, Order Lamniformes); c, *Centroscymnus coelolepis;* d, *C. crepilater*; e, *Centrophorus uyato*; f, *C. squamosus*; g, *Deania profundorum*; h, *Etmopterus pusillus* (c-h family Squalidae, Order Squaliformes); i, *Raja rhina* (Rajidae, Rajiformes); j, *Hydrolagus colliei* (Chimaeridae, Chimaeriformes). **B:** N-terminal sequence of the protein Pcon-Z3 from *Hydrolagus colliei* (a) compared with the sequence of the protein Pcon-Z3 from *Scyliorhinus canicula* (b) [42].

Source : MNHN, Paris

corresponding protein in *S. canicula* [42] (referred to as Z3 in the original works of GUSSE & CHEVAILLIER [18]).

Judging from the information available, it appears that chondrichthyans share a common SNBP pattern consisting of a very basic cysteine-lacking protein (Pcon-Z3) and a reduced set of cysteine-containing proteins (Pcon-Z1, Z2). It also appears that the amino acid composition and sequences of the SNBPs have undergone a remarkable divergence during the separation of holocephalans and selachians (Table 3 and Fig. 4B; see [37] for a more extensive discussion).

TABLE 3. — Amino acid composition (mol %) of proteins Pcon.Z1, Z2 and Z3 from *Hydrolagus colliei* (HC), and *Scyliorhinus canicula* (SC). Pcon.Z1, Z2 and Z3 compositions from *S. canicula* are from [8, 25, 42]. Res* = Total number of amino acid residues.

	Pcon-Z1		Pcon-Z2		Pcon-Z3	
	HC	SC	HC	SC	HC	SC
Lys	12.2	16.0	9.9	13.0	20.7	-
His	6.9	4.0	1.2	6.5	4.3	-
Arg	30.1	32.0	39.3	37.0	27.6	64.5
Asx	4.3	4.0	1.7	-	4.6	-
Thr	2.8	4.0	4.6	2.2	2.6	-
Ser	4.0	4.0	9.7	4.3	7.4	9.7
Glx	6.0	2.0	1.5	2.2	5.7	-
Pro	6.9	6.0	3.8	2.2	6.9	-
Gly	6.4	2.0	21.1	2.2	4.0	19.4
Ala	2.7	2.0	3.8	10.9	3.4	3.2
1/2 Cys	1.2	8.0	1.4	8.7	-	-
Val	3.5	4.0	-	4.3	1.9	-
Met	1.2	-	-	2.2	1.6	-
Ile	3.9	-	1.7	2.2	-	-
Leu	4.5	8.0	1.8	2.2	3.8	-
Tyr	2.2	2.0	-	-	-	3.2
Phe	1.2	2.0	2.3	-	5.3	-
Res*	79±4	50	66±2	46	45±4	31

SNBPs from Osteichthyes

We have studied a number of bony fish species [35, 38-39, 41] and have exhaustively reviewed the information on osteichthyan SNBPs [41]. The most striking feature that emerged from these analyses was the apparent lack of a unique protein pattern which could be considered representative of all the SNBPs of this group. With a few exceptions [23, 24], the SNBPs of fish belong to one of the five electrophoretic patterns shown in Fig. 5 [39]; namely a, presence of somatic histones and absence of any other sperm-specific nuclear protein (this is a similar situation to that already described in the agnathan *P. marinus*); b, presence of somatic histones but with a marked increase in the histone H1 content (in this case we will consider that the specific nuclear protein corresponds to the additional increase of the corresponding H1); c, presence of a histone complement coexisting with one additional SNBP belonging to PL type; d, presence of one (or few) PL proteins that completely replace histones in sperm nuclei; and e, presence of "typical protamines" which also replace completely somatic histones. There appear to be important differences between "typical protamines" and the SNBPs from groups b, c and d. The former

Source : MNHN, Paris

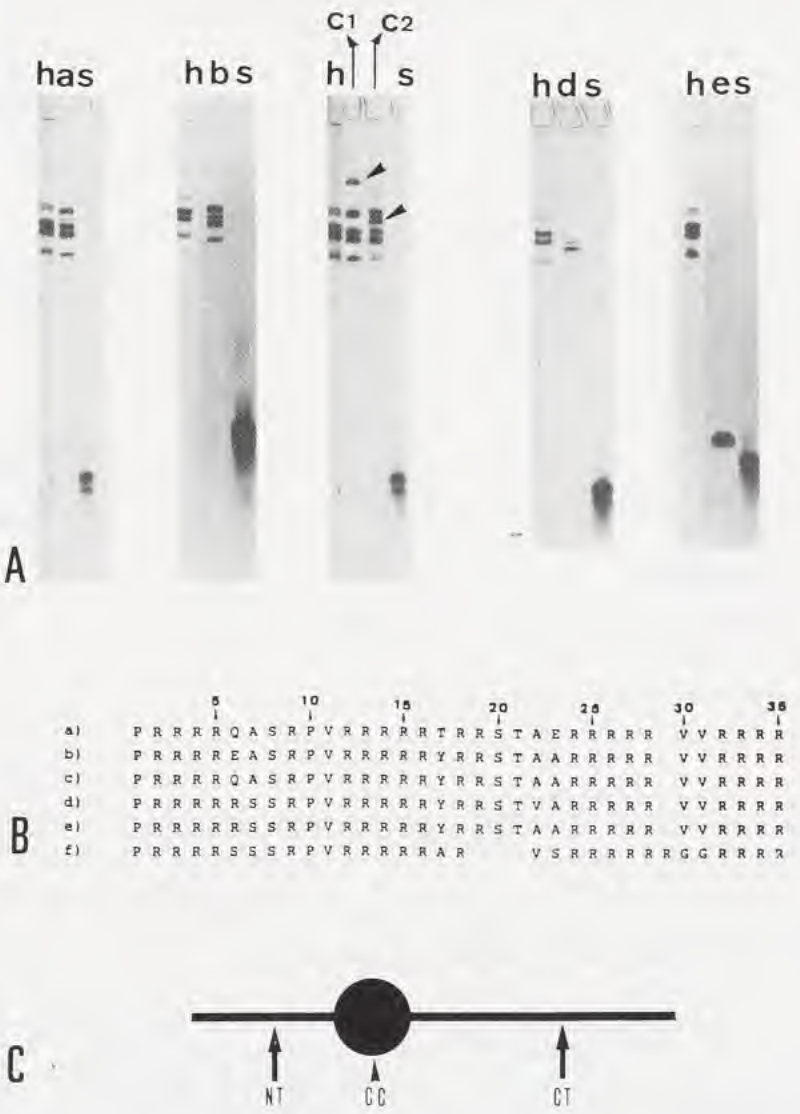


FIG. 5. — **A:** Several representative electrophoretic patterns of the main SNBPs from bony fish; a, somatic histones (*Trigla lucerna*, Triglidae); b, somatic histones with increased quantities of H1 (*Pagellus acarne*, Sparidae); c, somatic histones coexisting with an additional specific protein belonging to PL type (c1: *Merluccius capensis*, Merlucciidae, c2: *Cataetyx laticeps*, Bythitidae); d, protein PL which replaces histones in sperm nuclei (*Mullus surmuletus*, Mullidae); e, "typical" fish protamine (*Dicentrarchus labrax*, Percichthyidae). Chicken erythrocyte histones used as a standard (h) and commercial salmine (s) are shown for comparison in each electrophoresis. **B:** Amino acid sequence of some bony fish "typical" protamines [31, 38]. a, *Dicentrarchus labrax*; b-e, Tuna fish (*Thunnus thynnus*) b, fraction Y1, c, fraction Y2, d, fraction Z1, and e, fraction Z2; f, protamine 2b from rainbow trout (*Salmo irideus*). **C:** Schematic representation of the structural organizaton of PL from *M. surmuletus*; NT: 20 aa residues; CC: ~75 aa residues; CT: 80-85 aa.

are very small molecules consisting of a few amino acid types (see Table 4-e and *D. labrax* protamine sequence [38] in Fig. 5B). In contrast, the SNBP from the other groups are larger molecules, and exhibit a more complex amino acid composition which may be considered similar to that of the histones of the H1/H5-family, but with a higher arginine content (type PL). These

Source : MNHN, Paris

TABLE 4. — Amino acid composition (mol %) of the bony fish SBPs shown in figure 5; a) whole histones from *T. lucerna* sperm nuclei; b) H1 histone of *P. acarne*; c1) specific sperm protein of *M. capensis*; c2) SNBP of *C. laticeps*; d) main PL of *M. surmuletus*; e) "typical" protamine of *D. labrax*.
t = trace amounts

	a	b	c1	c2	d	e
Lys	16.7	21.7	17.9	19.3	24.2	-
His	1.6	-	-	6.5	-	-
Arg	7.9	3.5	19.2	10.4	22.1	61.8
Asx	3.3	4.5	2.0	3.6	5.1	2.9
Thr	4.9	2.9	6.9	5.4	4.5	5.9
Ser	5.0	8.4	6.9	8.4	9.2	5.9
Glx	6.2	6.4	1.3	5.4	t	2.9
Pro	5.7	10.9	14.6	7.8	6.8	5.9
Gly	9.3	11.7	7.7	6.3	5.4	-
Ala	13.4	18.1	14.5	7.1	11.9	5.9
1/2 Cys	t	-	-	-	-	-
Val	7.2	5.4	3.0	4.2	4.9	8.8
Met	t	t	-	3.6	t	-
Ile	5.5	1.1	0.6	3.2	t	-
Leu	9.7	4.6	4.3	6.6	5.8	-
Tyr	1.3	-	1.0	t	t	-
Phe	2.3	0.9	-	1.9	t	-

features are common to both the PL proteins that coexist with histones in the sperm nuclei (lanes b and c in Fig. 5A, and Table 4), and to those PL proteins that wholly replace histones, as occurs in the family Mullidae (Fig. 5A lane d, and Table 4). The electrophoretic pattern of the SNBPs of this family consists of two proteins of almost identical electrophoretic mobility and amino acid composition. They could possibly have arisen from a unique ancestor by a mechanism of gene duplication [38]. The partial sequence of one of them [35] indicates that it consists of approximately 180 amino acid residues organized in three structural domains (N-terminal, central core and C-terminal), as in H1 histones (Fig. 5C). The N-terminal region is 20 amino acid long (50% of them being basic residues) and contains the repetitive motive Ser-Pro-basic-basic which has been described in the N- and C-terminal regions of the sperm histones from echinoderms [33, 43]. The central core is trypsin-resistant and displays a high percentage of similarities with the equivalent region of H1 histones. The C-terminal zone is about 85 residues long and contains most (~70%) of the basic amino acid residues of the molecule.

DISCUSSION

Characteristics and distribution of SNBP molecules

Taking into account the structural and compositional features of the deuterostome SNBPs (other than histones), it is possible to group these molecules into two categories. I) proteins similar to histones (mainly to the H1 type), and II) highly specialized proteins.

The first group, referred to as PL (protamine-like) owing to its enhanced basicity, are relatively large proteins, with a complex amino acid composition similar to that of histone H1. All PL proteins studied to date share the same general molecular organization. All of them have a trypsin-resistant core consisting mainly of neutral amino acid residues, flanked by very arginine and/or lysine-rich N- and C-terminal domains. Depending on the species, PL proteins may be able

Source : MNHN, Paris

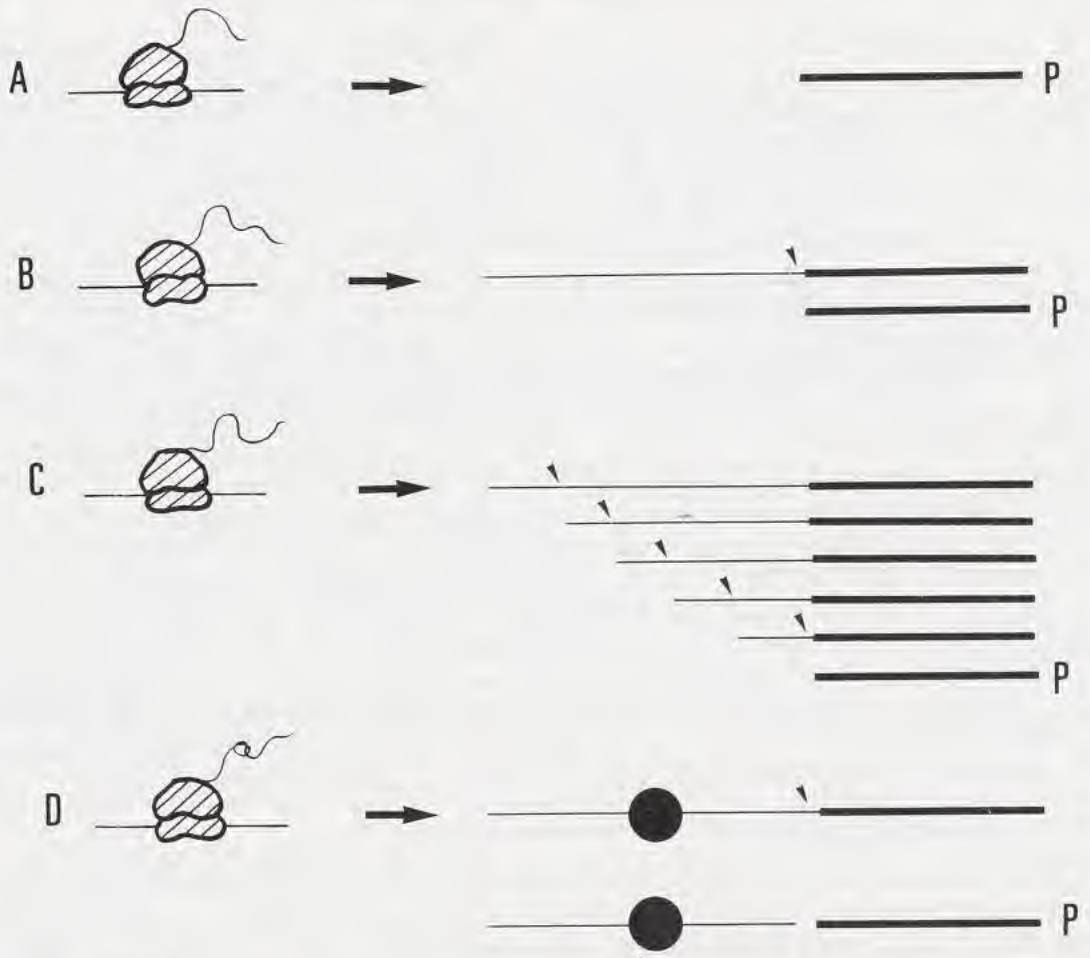


FIG. 6. — Different mechanisms of post-translational processing in SNBPs leading to specialized P proteins: **A:** Direct translation from mRNA; e.g. "typical" protamine of bony fish. [20]. **B:** Translation of a precursor which leads to the appearance of a P protein by a single proteolytic cleavage; e.g. "protamines" of cephalopod molluscs [46]. **C:** Translation of a precursor which undergoes several progressive cleavages. Each cleavage introduces important changes in the extent of chromatin condensation; e.g. "protamines" of neogastropod molluscs [4]; and the mammal P2 protamine [7]. **D:** Translation of a large PL protein. The C-terminal domain is cleaved to generate a very specialized P protein and a second PL protein; e.g. bivalve molluscs [5-6].

to replace somatic histones to different extents. Among the deuterostomes studied so far, PL proteins appear in all ascidiacean tunicates (PLasc) as well as in some fishes (*Mullus, Merluccius*, etc.). However, it is interesting to note that PL proteins are also present in protostomes such as in the case of PL-I and PL-II of bivalve molluscs [3] and "protamines" of patellogastropods and polyplacophorans [15, 16]. The origin of this type of protein is not well established. From the sequences published [3] and the ongoing analysis of the sequences of PLasc and PL from *Mullus* [35], it becomes apparent that the central core of these proteins shares an enormous similarity with the globular core of the histones of the H1 family. Therefore, PL proteins may be evolutionarily related to this histone. In contrast to the central core, the PL N- and C-terminal tails exhibit an enormous compositional variability. From the evolutionary point of view, there does not seem to exist a direct link among the zoological groups which display PL proteins. In our opinion, the PL sperm specific proteins may have appeared independently, several times during the evolution of

Source : MNHN, Paris

the animal kingdom (bivalves, polyplacophors, patellogastropods and tunicates, and several times among fish).

The proteins of the second type, which we refer to here as "very specialized proteins" (or "P" proteins), exhibit the following characteristics: they are short molecules (from approximately 30 to 120 amino acid residues) and they exhibit a very simple amino acid composition (with only a very few amino acid types). Arginine and/or lysine are present in a large amount (50%-80%) and hydrophobic residues are scarce or absent. Among the deuterostomes studied here, P proteins appear sporadically in tunicates (Psty.2 in genus *Styela*), in cephalochordates (Pceph), in chondrichthyan fishes (Pcon.Z3) and in osteichthyes (typical protamines). However, as occurs with PL proteins, P proteins are widely distributed over a broad spectrum of both phylogenetically distant and closely related groups. Thus P proteins have been described in bivalves [2], archaeogastropod molluscs [13-14, 16-17], neogastropods ("ripe" protamines) [4], cephalopod molluscs [46], crustaceans [11], echinoderms (Φ_0 protein) [34] as well as in fish, amphibians, birds and mammals [22, 31, 47]. These P proteins are not always homologous and it is reasonable to think that they have also appeared independently several times and from several different cellular processes (Fig. 6) during the course of evolution. In the case of bivalve molluscs, it has been demonstrated that some "specialized proteins" (PL-IV in the original works of CARLOS *et al.* [5-6]) actually correspond to the C-terminal region of a larger protein precursor belonging to the PL type (referred to as PL-I by some authors) (see also AUSIO, this volume). They appear in the nuclei following a precise proteolytic cleavage (Fig. 6D). From this perspective, the similarity between the amino acid composition of the Psty.2 protein and the C-terminal part of PLasc (Table 1) is very suggestive of a possibly related cleavage post-translational mechanism in the case of these proteins. It seems possible that some P proteins have arisen from a simplification (genotypical or phenotypical) of other proteins of the PL type, although other origins for particular P proteins (as is the case of Pcon.Z1, Z2) can not be disregarded.

General evolutionary considerations

From the information presented in this chapter, it is quite evident that it is difficult to trace an uninterrupted evolutionary line connecting the different SNBPs of deuterostomes. The proposition of an evolutionary link between histones and protamines which has been observed in different phylogenetic groups (histone H1 → PL proteins → very specialized P proteins; see also AUSIO, this volume) has most likely occurred independently in each of them. Another important consideration to be made arises from the fact that when a group consisting of a large number of species is analyzed, the evolutionary trend within this group may be masked by the variability of SNBPs present in the limited sample of species analyzed. This is for instance the case in bony fish where the appearance of "protamines" seems to be sporadic. Two hypothesis have been put forward to explain the apparent random distribution of SNBPs within this group. The first one proposes a mechanism of horizontal evolution of the genes of the fish protamines. In the second alternative, the phenomenon is explained by the loss of the expression of these genes during the formation of some groups of fish [21, 41]. It is important to stress the fact that fish protamines, which for historical reasons have long been considered to be the "typical protamines", in fact represent only one of the many types of the highly specialized P proteins. Fish protamines do not necessarily represent the final goal of SNBP molecular evolution. Yet, they are of special interest because of the extent of similarity they share with the P1 protamine ancestors of tetrapods [31].

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Source : MNHN, Paris

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Source : MNHN, Paris

Heparin-Binding Proteins on Bull, Boar, Stallion, and Human Spermatozoa

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ABSTRACT

The seminal plasma from many mammalian species contains heparin-binding proteins which bind to the sperm surface at ejaculation and appear to mediate sperm capacitation modulated by glycosaminoglycans (GAG) present in the reproductive tract of the female. The molecular mechanisms underlying this process is only poorly understood, however. Furthermore, seminal plasma heparin-binding proteins have been structurally characterized in only few mammalian species. Here, we summarize our present knowledge on sperm surface-associated heparin-binding proteins in four different mammals: boar, bull, stallion and man. The major conclusion of this comparative study is that the major heparin-binding proteins from these species belong to only three different protein families. The relative abundance of proteins of different families on spermatozoa from the different species, together with structural diversity between proteins of the same family in different species, and species-specific topographical localization of homologous heparin-binding proteins on spermatozoa, may contribute to the specific spermatozoon phenotype, which in turn may modulate species-specific effects of GAGs on sperm capacitation.

RÉSUMÉ

Les protéines liant l'héparine dans les spermatozoïdes du Taureau, du Verrat, du Cheval et de l'Homme

Le plasma séminal de nombreuses espèces de mammifères contient des protéines liant l'héparine qui se lient à la surface des spermatozoïdes à l'éjaculation et semblent être les médiateurs de la capacitation des spermatozoïdes, qui est modulée par les glycoaminoglycanes (GAG) présents dans le tractus génital de la femelle. Toutefois, les mécanismes moléculaires sous-jacents à ce processus sont mal connus. De plus, la structure des protéines du plasma séminal liant l'héparine a été caractérisée seulement dans quelques espèces animales. Nous résumons ici l'état actuel des connaissances sur les protéines liées à l'héparine associées à la surface des spermatozoïdes chez quatre mammifères: Verrat, Taureau, Cheval et Homme. La conclusion principale de cette étude comparative est que les protéines majeures liant l'héparine de ces espèces appartiennent à seulement trois familles différentes de protéines. L'abondance relative des protéines de différentes familles sur les spermatozoïdes des différentes espèces, ainsi que la diversité structurale entre les protéines d'une même famille dans les différentes espèces, et que la localisation topographique spécifique à l'espèce des protéines homologues liant l'héparine sur les spermatozoïdes, peuvent contribuer au phénotype spécifique du spermatozoïde, qui ensuite module les effets spécifiques des glycoaminoglycanes sur la capacitation des spermatozoïdes.

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Source : MNHN, Paris

Following the differentiation of the haploid spermatozoon, the cell is released from the epithelium of the seminiferous tubules passing from the testis to the epididymis. Mammalian spermatozoa are highly differentiated by the time they leave the testis. Nonetheless, at this stage, they do not have the ability to fertilize eggs. Spermatozoa gain this capability while passing through the epididymis (epididymal maturation) and/or after residing in the female tract for some period of time (capacitation) (reviewed in [35]). Though our knowledge of the molecular basis of these processes is still in its infancy, one of the most prominent, and best documented, physiological changes in the spermatozoa is a continous remodelling of their plasma membrane components as spermatozoa travel through the various microenvironments within the male and the female genital tracts [17, 35]. Both membrane-integrated and surface-adsorbed components (lipids or proteins) either change their location in or on the plasma membrane, are altered, masked, or replaced as a response to the constantly changing osmolarity and chemical composition of the millieu surrounding the sperm. In particular, at the time of ejaculation, spermatozoa from the distal (cauda) epididymis are mixed with secretions of the male accessory sexual glands. Recent evidence suggests that components of seminal fluid upon attaching to the sperm surface may regulate important sperm functions: some (glyco)proteins stabilize the plasma membrane and may prevent premature acrosome reactions, and others are believed to mediate interactions between spermatozoa and the zona pellucida. Here, we will restrict the discussion to the structure and proposed biological role of heparin-binding proteins of the seminal plasma of different mammals: boar, bull, stallion and man. Interestingly, the seminal plasma heparin-binding proteins of these four species belong to only three different protein families, which include both capacitation factors and carbohydrate-recognition (putative zona pellucida-binding) molecules. Differences between species in the relative abundance and topography of proteins from different families on the sperm surface, may contribute to the species-specific physiology of mammalian spermatozoa.

RESULTS AND DISCUSSION

Heparin-like GAGs and heparin-binding proteins in fertilization

Proteoglycans with heparin- and chondroitin sulphate-like glycosaminoglycan side chains are secreted by the epithelium of the female reproductive tract, particularly at high concentration during the follicular phase of the estrous cycle, and have been shown to specifically invoke sperm capacitation in a number of mammalian species, as measured by the onset of agonist-inducible acrosome reactions [32]. Thus, *in vitro* incubation of epididymal sperm from such different mammalian species as bovine, hamster, human, rabbit, or equine, with glycosaminoglycans significantly accelerated the development of the sperm capacitated state as judged by their increased ability to fertilize homologous eggs. In addition, since exposure of bovine epididymal spermatozoa to seminal plasma from the same species enhanced their ability to undergo the acrosome reaction in the presence of heparin [25], it follows that the effects of heparin-like GAGs appear to be mediated by seminal plasma heparin-binding proteins which coat the sperm surface at ejaculation.

Seminal plasma heparin-binding proteins on bovine spermatozoa

Comparison of the heparin-binding protein composition of epididymal and ejaculated spermatozoa showed the presence of a 30 kDa and several 15-17 kDa proteins in the ejaculated spermatozoa [10]. The same group of proteins were adsorbed to the sperm surface following exposure of epididymal sperm to seminal plasma [24]. The 30 kDa protein has not been structurally characterized. The primary structures of the 15-17 kDa proteins, designated BSP-A_1, BSP-A_2 and BSP-A_3, have been reported [16, 30]. BSP-A_1 and BSP-A_2 have the same polypeptide backbone but the former contains a single O-glycosylated trisaccharide (NeuNAc α(2-6)-Gal ß(1-3)-GalNAc-) attached at threonine-11 [5], and together they are also known as Major

Source : MNHN, Paris

Protein (MP) or PDC-109 (Fig. 1A). Structurally, BSP-$A_{1/2}$ and BSP-A_3 are made up of two tandemly-arraged homologous modules of around 40 residues that share the consensus sequence of the collagen- and heparin-binding type-II modules of fibronectin [30] (Fig. 1A). Each of these repeats contains the amino acid residues which have been identified by alanine scanning mutagenesis to be critical for collagen binding [2]. In addition, the isolated B-domain of PDC-109 retained affinity for immobilized collagen [2]. Recently, a refined H^1-NMR-solution structure of the 45 -residue PDC-109 domain B has been reported [12]. However, the folding pattern of the whole molecule and the heparin-binding site(s) remain to be established.

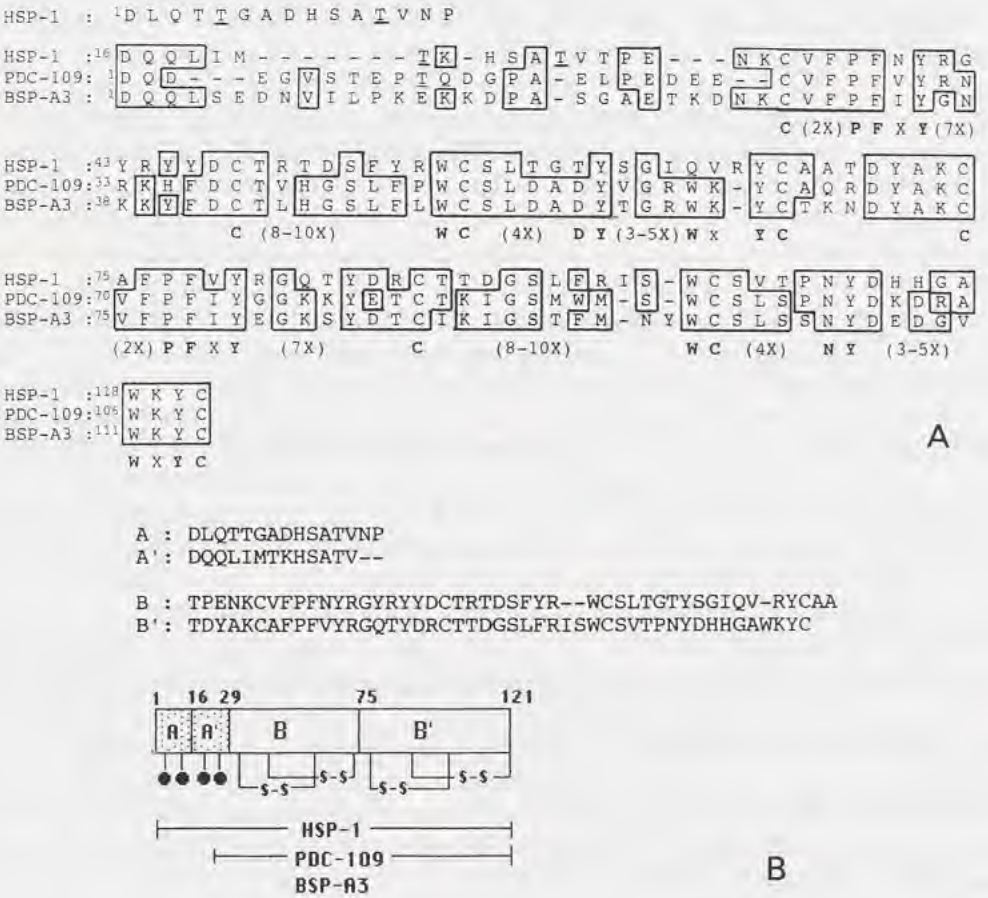

Fig. 1. — **A**: Comparison of the primary structure of the major heparin-binding proteins from stallion (HSP-1) and bovine (PDC-109 and BSP-A3) seminal plasma. Identical residues in at least two proteins are shown in boxes. The consensus amino acids of the fibronectin type II module and the spacing between them are shown below the sequence alignment. T, O-glycosylated threonine residues. **B:** Amino acid sequence alignment of the A- and B-type internal repeats of stallion heparin-binding protein HSP-1, and a scheme of the domain arrangement in HSP-1 and in bovine PDC-109 and BSP-A3. –•, indicates the relative positions of O-glycosylation sites; S-S, disulphide bridge.

Proteins cross-reacting with monospecific antibodies against either BSP-$A_{1/2}$, BSP-A_3, or BSP-30 kDa have been detected in the seminal fluids of human, porcine, hamster, mouse, and rat [18]. However, only the gene expression of bovine BSP-$A_{1/2}$ has been studied [29]. Southern

Source : MNHN, Paris

blot analysis of genomic DNA indicated that BSP-A_1/A_2 is coded for by a single gene per haploid bovine genome [29]. The protein is secreted by the seminal vesicle epithelium at a concentration of 16-25 mg/ml [5, 29].

The topographical localization, the nature of the sperm surface acceptor molecules of PDC-109, and its physiological role are controversial. Thus, AUMÜLLER *et al.* [1] reported that PDC-109 binds preferentially to the middle piece and neck region of bovine spermatozoa and identified by immunoblotting analysis a 65-67 kDa protein duplet as a PDC-109 acceptor site on epididymal spermatozoa. These authors postulated that binding of PDC-109 to its acceptor site might be regarded as a physiological event related to the onset of hyperactivated sperm motility [1]. On the other hand, Manjunath et al. have shown that isolated PDC-109 possesses the ability of binding a number of ligands, such as apolipoprotein A-I (apoA-I) and apoA-I associated high-density lipoproteins, different types of collagen (I, II, IV, and V), fibrinogen, heparin, calmodulin, phospholipase A_2 (PLA_2), and phosphorylcholine-containing lipids [23]. Using immunofluorescence, MANJUNATH *et al.* have reported that BSP-$A_{1/2}$ is located on the entire sperm head surface, "although the intensity was noticeably stronger at the midpiece region" [22]. These authors have proposed that BSP-$A_{1/2}$ may play an important role in sperm lipid modification that occurs during capacitation and the acrosome reaction. In their mechanistic theory, binding of BSP-$A_{1/2}$ to choline phospholipids on the sperm surface may block PLA_2 from acting on these phospholipids and prevent sperm from undergoing a premature acrosome reaction. In addition, acting as decapacitation factors sequestering cholesterol and choline phospholipid, BSP-$A_{1/2}$ may alter the fluidity and permeability of the spermatozoal membrane and allow calcium to enter for activation of PLA_2, which would then produce lysophospholipids that are known to destabilize membranes and trigger membrane fusion (acrosome reaction).

In addition to the proposed role in membrane lipid remodelling events, MANJUNATH *et al.* [22] have reported a specific interaction of BSP-$A_{1/2}$, BSP-A_3, and BSP-30 kDa with insulin-like growth factor-II (IGF-II), and hypothesized that BSP-proteins bound to the cell surface could modulate the IGF-II action by serving as carriers as well as cell surface binding site for the hormone.

Our own data showing that, upon mixing of spermatozoa with seminal plasma at ejaculation, 9 million molecules of PDC-109 on average become coated to the sperm surface, and that this figure decreases only to 8 million molecules per spermatozoon after incubation for 24 h in capacitation medium at 39° C [5], do not support a role for BSP-$A_{1/2}$ as a decapacitation factor. Furthermore, BSP-$A_{1/2}$ eluted from a Sephadex G-200 gel filtration column as aggregated molecules with an apparent molecular mass of 60-120 kDa [22], indicating that in addition to binding to 65-67 kDa acceptor protein(s) and choline phospholipids, BSP-A1/2 becomes coated to the sperm surface as a multimer. How the aggregation state of BSP-A1/2 affects the various ligand-binding activities reported in *in vitro* systems deserves further investigation. Clearly, much work is needed to establish the actual biological function(s) of, and the mechanisms used by, the different members of the bovine seminal plasma (BSP) protein family on sperm physiology.

Another abundant heparin-binding component of bovine seminal plasma (~7 mg/ml) is acidic seminal fluid protein (aSFP) [15]. Deduction of the primary structure of aSFP from a complete cDNA clone [34] showed that aSFP shares 43% sequence identity with porcine spermadhesins (see below) (Fig. 2). On average, 6 x 10^6 molecules of aSFP bind to a narrow region on the apical part of the acrosome of an ejaculated spermatozoon, but this amount decreases to undetectable levels in capacitated sperm [14]. This indicates that aSFP might have a role as a decapacitation factor, and shows that proteins from the same family (spermadhesin) play different functions in the fertilization process of different mammalian species, i.e. in bull and boar (see next section).

Source : MNHN, Paris

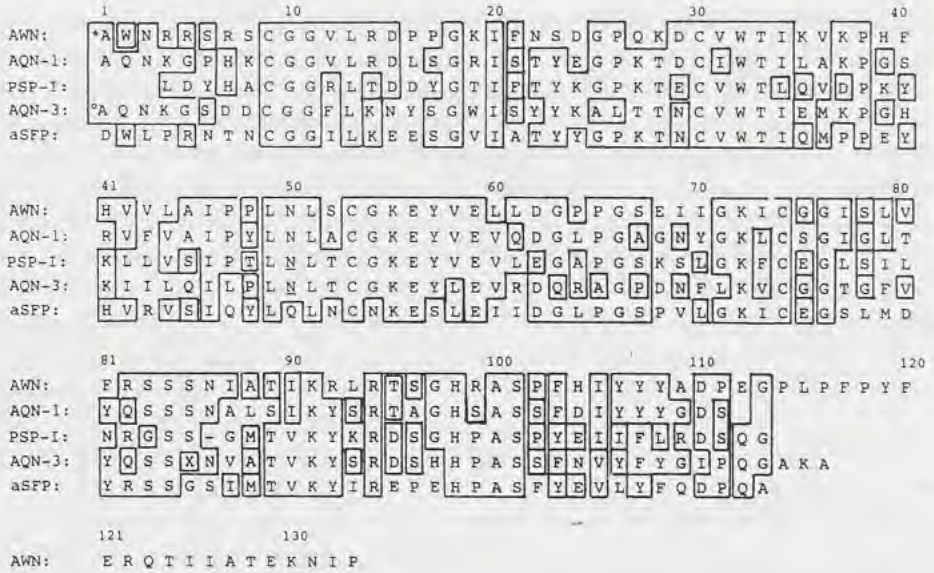

FIG. 2. — Alignment of the amino acid sequence of boar spermadhesins AWN, AQN-1, PSP-I, and AQN-3, and the bovine seminal plasma polypeptide aSFP. Identical residues within at least two of these proteins are shown in boxes. *, this residue is acetylated in AWN-1; °, AQN-3 also exists as a glycosylated form; N, is the conserved glycosylation point in PSP-I and the AQN-3 isoform. Two disulphide bridges between nearest-neighbour cysteine residues are conserved within the boar spermadhesins.

The major boar sperm-associated heparin-binding proteins belong to the spermadhesin family

The boar seminal plasma and sperm-associated heparin-binding proteins have been isolated using affinity chromatography on Sepharose-heparin [27]. A minor 18 kDa glycoprotein with an N-terminal amino acid sequence DQHLPGRFLXPAITSDDKCVFPFIYKGNL... was characterized [27]. This clearly showed that boar spermatozoa also possess a heparin-binding protein of the same protein family as the bovine polypeptides. However, the major heparin-binding components, designated AQN-1, AQN-2 (PSP-I), AQN-3, AWN-1, and AWN-2 belong to a novel protein family for which the term "spermadhesin" has been coined (reviewed in [6]). The nomenclature used for boar spermadhesin was based on the first three amino acid residues of their sequence, alanine(A)-glutamine(Q)-asparagine(N) and alanine(A)-tryptophan(W)-asparagine(N); a number indicates the reverse-phase HPLC elution order of polypeptides containing the same N-terminal sequence. These proteins are 111-133 amino acid long, contain two conserved disulphide bridges between nearest-neigbor cysteine residues, and have 40-60% sequence identity (Fig. 2). Posttranslational modifications contribute to the diversity of the family: AWN-2 is identical as AWN-1 but has an acetylated N-terminal alanine residue; PSP-I is constitutively glycosylated; AQN-3 and AWN (isoforms 1 and 2) are found as both non-glycosylated and as N- and O-glycoforms. On the other hand, no glycosylated isoforms of AQN-1 have been reported.

Spermadhesins were first identified by their carbohydrate-binding, and zona pellucida-binding capabilities [6]. Subsequently, it was shown that, in addition to their lectin-like activity, boar spermadhesins are multifunctional proteins which combine within the same molecule heparin- (AQN-1, AQN-2, AQN-3, and AWN) and/or serine proteinase inhibitor- (AQN-1 and AWN) binding abilities. It has been proposed that serine proteinase once bound to sperm surface acceptor molecules, may stabilize or protect sperm surface membrane specific sites for sperm-egg interaction [26]. Inhibitors are then released from the sperm surface during sperm residence in the

Source : MNHN, Paris

female genital tract allowing zona pellucida-binding sites to become exposed. Therefore, the spectrum of ligand-binding strongly suggests that boar spermadhesins may play a role in at least two important aspects of fertilization, sperm capacitation and gamete interaction.

The major biological source of spermadhesins is the secretion of the seminal vesicle epithelium, where the concentration of different spermadhesins ranges from 0.6-7.2 mg/ml [13]. However, AWN-1 is also synthesized by the tubuli recti and the rete testis [31] and is the only spermadhesin found on the surface of epididymal sperm. The amount of coated AWN-1 (5.9-7.5 x 10^6 molecules/epididymal spermatozoon) is sufficient to cover one-third of the entire surface of the sperm head, i.e. the acrosomal cap, with a one-molecule-thick layer. We hypothesize that AWN-1 may be one of the factors contributing to the fertilizing activity of epididymal spermatozoa. Following ejaculation, 12-60 x 10^6 molecules of each AQN-1, AQN-2, AQN-3, and extra 50 x 10^6 AWN molecules (isoforms 1 and 2) become adsorbed on the apical third of the acrosomal cap of spermatozoa, the place where porcine sperm initiate binding to the zona pellucida of the oocyte. However, approximately 60% of adsorbed spermadhesins AQN-1, AQN-2, and AQN-3 are released after 3 h *in vitro* capacitation, the amount of AWN-1 decreases to the level found on epididymal sperm, and the whole AWN-2 population is lost. This figure changes only slightly upon 24 h capacitation.

Spermadhesins AWN-1 and AQN-3, as monomers, possess binding affinity for phosphorylethanolamine (PE) matrices, and the PE-binding site is different from the carbohydrate-recognition domain (unpublished results). Phosphorylethanolamine is a major substituent of boar sperm membrane phospholipids [33]. This suggests that AWN-1 and AQN-3 may bind directly to sperm membrane lipids while the other spermadhesin moieties may coat on top of them as aggregated molecules, and indicates that different subpopulations of spermadhesins may play diverse roles as either decapacitation or acrosome stabilizing factors, positive capacitation elements, and/or receptors for zona pellucida.

Glycosylated isoforms of spermadhesins AQN-3, PSP-I, and AWN bind heparin but fail to bind zona pellucida glycoproteins and soybean trypsin inhibitor [7, 8, 9]. Therefore, attachment of a glycosyl moiety may modulate the receptor function of spermadhesins isoforms, i.e. switching the receptor function between a capacitation factor (heparin-binding) and a primary zona pellucida-binding molecule. The functional inability of glycosylated spermadhesins has been found to be due to steric blockade of the ligand-binding site which results from attachment of a single oligosaccharide to each glycoform, either N-linked to Asn^{50} or O-linked to Ser^{52} or Thr^{95}. This indicates that the zona pellucida- and the inhibitor-binding sites may be located around the glycosylated residues and are different from the heparin-binding site. AWN possesses a consensus sequence for heparin-binding (^{3}NRRSRS^8) which, in a recently developed three-dimensional model for spermadhesins [6], is located at the opposite side of the proposed zona pellucida/inhibitor binding domain. These arrangement of binding domains would be in agreement with the experimental results. Nevertheless, the exact three-dimensional structure and epitope topography of spermadhesins, as well as the way in which the heparin-like glycosaminoglycan- and zona pellucida glycoprotein-binding information is transduced, require further investigation.

Stallion spermatozoa possess heparin-binding proteins of both the BSP and the spermadhesin family

The heparin-binding proteins on ejaculated stallion spermatozoa have recently been isolated and structurally characterized [4]. The major components, termed HSP-1 and HSP-2, belong to the same protein family as bovine BSP- ($A_{1/2}$ and A_3). We estimate that, together, HSP-1 and HSP-2 may account for over 70% of the total sperm-associated heparin-binding proteins.

The primary structure of HSP-1 has been completed [3]. Interestingly, it is a mosaic protein which consists of 121 amino acid residues organized in two types of homologous repeats arranged in the pattern AA'BB' (Fig. 1B). Each of the N-terminal 13-15 residues long A-type

Source : MNHN, Paris

repeat (residues 1-15 and 16-28) contains two O-linked oligosaccharide chains each (threonine residues 5, 12, 22, and 27) and they have 47% sequence identity. The B-type repeats span 44-47 amino acids each (residues 29-72 and 75-121), are not glycosylated, show 43% sequence identity, possess two disulphide bridges (between cysteines, 34-58, 48-72, 80-106, and 94-121) and have the consensus pattern of the fibronectin type-II module. The sequence of the A' domain can only be aligned with the bovine proteins by introducing numerous gaps and the N-terminal A-domain is absent in bovine BSP-$A_{1/2}$ (PDC-109) and BSP-A_3 (Fig. 1A). In addition, only the glycosylation site at threonine 22 is conserved in BSP-$A_{1/2}$. On the other hand, the B-domains are highly conserved in bovine BSP-$A_{1/2}$ and BSP-A_3 proteins (Fig. 1A). However, the critical residues for gelatin-binding are only conserved in the second (B') repeat. Altogether, it seems reasonable to propose that the different combination of structural domains of the bovine and equine BSP-proteins could confer species-specific properties on a common heparin-binding polypeptide framework. Further characterization of the topographic localization, sperm surface acceptor molecules and coating dynamics, may help to clarify this point.

Another abundant sperm-associated heparin-binding protein is a 16 kDa component which may represent some 20% of the total stallion sperm-associated heparin-binding proteins. This 16 kDa protein is not glycosylated and is immunologically related to boar AWN spermadhesin [4]. In addition, initial structural characterization of this stallion AWN-protein shows that it shares a high degree of amino acid sequence with the boar protein (unpublished results). However, whereas the boar AWN spermadhesin binds homogeneously to the whole acrosomal cap surface [28], an indirect immunofluorescence study showed that the topographical localization of the 16 kDa AWN-related protein is restricted to the equatorial segment in specimens of both epididymal and ejaculated stallion spermatozoa [4]. AWN is the only member of the equine spermadhesin family found on either seminal plasma or spermatozoa.

In addition to proteins of the BSP and spermadhesin families, a minor component (25 kDa), which probably represents less then 10% of the total heparin-binding proteins, with the N-terminal amino acid sequence IIGGWEXEKHSKPWQVAVYHQGHFQXG..., has also found associated with ejaculated stallion spermatozoa. This sequence shows a high degree of sequence identity with serine proteinases of the kallikrein (EC 3.4.21.35) family, in particular with human prostate-specific antigen, PSA (27 kDa) [20], and with the androgen-dependent arginine-esterase (EC 3.4.21.34) mRNA product of canine prostate [11]. Human PSA is one of the enzymes involved in cleaving structural proteins of the seminal coagulum [19]. Its function in stallion sperm physiology has not been assessed, and deserves further study.

Heparin-binding proteins on human sperm: kallikrein and spermadhesins

Recently our laboratories have begun to characterize the human seminal plasma heparin-binding proteins which bind to the sperm surface. Using affinity chromatography, two major components (80 kDa and 28 kDa) have been isolated. Each protein accounts for around 50% of the total heparin-binding proteins in seminal plasma. The 28 kDa protein contains an amino acid sequence identical with previously characterized human plasma (glandular) kallikrein. The structure of the 80 kDa component(s) remains to be established. In addition, using indirect immunofluorescence microscopy, we have localized the binding site of an AWN-crossimmunoreacting protein to the equatorial segment of ejaculated human spermatozoa. Its relative abundance on spermatozoa has not been studied, however. Although the biological significance of these human molecules on sperm function remains obscure, we are currently elucidating the primary structure of human AWN as a first step towards determining its function.

Conclusion

The mechanism by which heparin modulates capacitation and/or the acrosome reaction is poorly understood. A major event in capacitation is believed to be the removal or alteration of a

Source : MNHN, Paris

protective coat from the sperm plasma membrane. This structural alteration may correlate with loss or reduction of plasma membrane cholesterol, uptake of extracellular calcium, and elevation of the internal pH, all of which constitute important steps in the capacitation and early steps of the acrosome reaction. The biological effects of heparin is not absolutely conserved in all mammalian species. Since, in bull, boar, stallion, and man, the seminal plasma heparin-binding proteins which become bound to the sperm surface belong to only three different protein families (Table 1), our working hypothesis is that species-specific modulation of sperm capacitation/acrosome reaction exerted by heparin might be related to a combination of , at least, the following factors:

The chemical structure, amino acid sequence and posttranslational modifications, of the heparin-binding proteins bound to the sperm surface;

The relative abundance of members of the different heparin-binding protein families in the seminal plasma;

The topography and amount of the different heparin-binding proteins on the surface of spermatozoa during capacitation.

TABLE 1. — Relative distribution of heparin-binding proteins of different protein families on mammalian spermatozoa. NF, not found.

Species	Protein family		
	BSP	Spermadhesin	Kallikrein
Bovine	++++	NF	NF
Pig	+	++++	NF
Horse	+++	++	+
Human	NF	++	++

To dissect the relative biological relevance and synergy of these, and possibly other, factors in heparin-mediated sperm capacitation is one of the main goals of our laboratories.

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Source : MNHN, Paris

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Source : MNHN, Paris

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Source : MNHN, Paris

Histone Gene Expression During Mammalian Spermatogenesis: Structural and Functional Aspects

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ABSTRACT

The chromatin of spermatogenic cells undergoes structural rearrangements upon differentiation from spermatogonia to mature spermatozoa. During the haploid stages of mammalian spermatogenesis, histones are gradually replaced first by transition proteins and then by protamines. The histone fraction in chromatin of spermatogenic cells is composed of testis specific subtypes as well as such histone isoforms, which are also found in somatic tissues. The subtype patterns of all histone classes except H4 change in a stage specific manner during mammalian spermatogenesis. This implies that control mechanisms exist which regulate the cell type specific expression of the individual histone subtype genes. This control may be exerted at the transcriptional level as exemplified by functional studies at the H1t promoter. Regulation also may take place posttranslationally as demonstrated by the polyadenylation of part of the mRNA of spermatogenic cells.

RÉSUMÉ

Expression des gènes des histones pendant la spermatogenèse des Mammifères: aspects structuraux et fonctionnels

La chromatine des cellules spermatogénétiques subit des ré-arrangements structuraux pendant la différenciation progressant de la spermatogonie au spermatozoïde mûr. Pendant les stades haploïdes de la spermatogenèse des Mammifères, les histones sont remplacées graduellement d'abord par des protéines de transition puis par des protamines. La fraction des histones dans la chromatine des cellules spermatogénétiques est composée de sous-types spécifiques du testicule ainsi que d'isoformes des histones qui sont aussi rencontrées dans les tissus somatiques. Les sous-types de toutes les classes d'histones sauf H4 changent spécifiquement en fonction des étapes de la spermatogenèse des Mammifères. Ceci implique qu'il existe un mécanisme de contrôle, qui régule l'expression spécifique à chaque type cellulaire des gènes individuels de chaque sous-type d'histone. Ce contrôle peut être exercé au niveau transcriptionnel comme l'ont montré les études fonctionnelles sur le promoteur des H1t. La régulation peut aussi être post-traductionnelle ainsi que le montre la polyadénylation d'une partie des ARNm des cellules spermatogénétiques.

Histones are the basic chromosomal proteins of eukaryotic organisms. The histone protein familiy is composed of five protein species which have been classified on the basis of size and function. First, five different classes were defined by electrophoretic means and were termed H1,

DOENECKE, D. & DRABENT, B., 1995. — Histone gene expression during mammalian spermatogenesis: structural and functional aspects. *In*: JAMIESON, B. G. M., AUSIO, J., & JUSTINE, J.-L. (eds), Advances in Spermatozoal Phylogeny and Taxonomy. *Mém. Mus. natn. Hist. nat.*, **166** : 525-535. Paris ISBN : 2-85653-225-X.

Source : MNHN, Paris

H2A, H2B, H3 and H4 (for reviews, see [93, 95]). Two copies of each of the four histones H2A, H2B, H3 and H4 form the nucleosomal core. Therefore, they are summarily described as *core histones* in contrast to the H1 class proteins, which interact with the linker DNA connecting nucleosomal cores and are termed *linker histones*. Core and linker histones have been detected in nearly all eukaryotes [95]. The yeast *Saccharomyces cerevisiae* is an exception in having no linker histone [18], but its chromatin forms core nucleosomes and shows a subunit pattern with a regular spacing [90].

Histone protein patterns have been monitored during spermatogenesis in a broad spectrum of lower and higher eukaryotes [6, 7, 13, 16, 54, 62, 66, 67, 83, 85, 87]. Our group has concentrated on mammalian systems and has studied the structure and expression of somatic and testis-specific histone genes from man and mouse [2-4, 31, 32, 34-38]. In this contribution, structural and functional features of testicularly expressed histone genes and gene products will be discussed in relation to different stages of spermatogenesis (see Table 1).

TABLE 1. — Mammalian spermatogenic histone gene expression. Compilation of histone subtype proteins detectable at specific stages of sperm differentiation before replacement by transition proteins and finally by protamines (data from rat, mouse and man or from one or two only of these). Asterisks indicate expression data obtained using gene probes.

Histone subtype	Cellular stage of histone detection	Reference
H1a-e	any stage (predominantly H1a*,H1c) in part replaced by H1t (in pachytene spermatocytes) no H1 left in late spermatids	12, 13, 15, 33*, 62, 83, 85
H1°	spermatogonia* (then decreasing)	42*
H1t	pachytene spermatocytes* replacing main type H1 (a, c)	35*, 50*, 59, 60, 67
H2A	any stage (H2A.1>H2A.2), in part replaced by TH2A H2A subtype encoded by poly(A)$^+$-mRNA in spermatids*	12, 67 71*
H2A.X	spermatogonia*	64*, 67
H2A.Z	spermatogonia (low), slight increase in pachytene spermatocytes	53, 67
TH2A	pachytene spermatocytes* partly replacing H2A	56*, 63, 67, 69
H2B	any stage, major part replaced by TH2B specific subtype in spermatids* (extended C-terminus)	12, 67 70*
TH2B	pachytene spermatocytes* (and later stages)	12, 57, 58*
H3	any stage (H3.2>H3.1)	12, 67
TH3	spermatogonia, absent during later stages	89
H3.3	any stage until spermatids, predominantly in spermatogonia	9, 67, 91
H4	any stage H1t associated H4* gene transcribed in pachytene spermatocytes	12, 67, 68 94*

Source : MNHN, Paris

OBSERVATIONS AND DISCUSSION

Testicular histone subtypes

The H1 linker histone family of mammals comprises several variant isoforms. The most detailed analysis of the H1 complement has been done in human and murine chromatin in several cell and tissue types. In humans, five main type H1 histone genes have been described [3] in addition to the gene encoding H1° [31], which is a histone confined to highly differentiated cell types, and the highly conserved H1t gene [34], which is only expressed in testicular cells. H1t protein sequences are known from man [34], other primates [59], mouse [35], rat [22] and boar [21]. The H1t protein is confined to male germ cells and is not a general meiosis specific variant [65]. As in humans, five different mouse H1 proteins (or genes) [3, 33, 62, 88, 89, 97] plus H1° [4] and H1t [35] were described. As yet, only one rat main type H1 gene [23, 36], the H1t gene [22] and a partial H1° cDNA sequence [17] have been described. The rat H1t gene and gene promoter structures have been intensely studied [49-51] and will be discussed below. The H1t fraction of the overall testicular H1 complement amounts to about 25% [78], and somatic type isoforms [62] and the H1° fraction [84, 87] constitute the remaining part of the H1 histone moiety in testicular chromatin. In pachytene spermatocytes and later stages H1t may comprise a much higher fraction [13, 67].

Core histone isoforms, which are restricted to testicular cells, have been described in several species. A pair of testicularly expressed genes consisting of an H2A and an H2B gene was described by HUH *et al.* [55]. These genes appear to code for the previously described testicular subtypes TH2A and TH2B, respectively [10, 58, 92, 98]. In addition to main type H2A and H2B isoforms, two H2A subtypes, which are replication independent, i.e. H2A.Z and H2A.X [53, 64], have been described. H2A.X is enriched in testicular chromatin, whereas H2A.Z is uniformly found in most somatic tissues [53]. A testicular subtype of H3 (TH3) has been isolated from rat testis [91]. Its unique amino acid composition (including three cysteine residues) indicates structural differences compared with all other known H3 subtypes, but as yet no TH3 gene has been identified in any mammalian genome. The replication independent H3 subtype H3.3 is also expressed during spermatogenesis. For example, H3.3 has been observed in spermatid stages of spermiogenesis [69], but it is also present at earlier stages of spermatogenesis [67, 91]. H4 is the most conserved of all histone classes. Its 102 amino acid sequence is strictly maintained in all mammalian species. This even applies to H4 genes which are differentially expressed. For example, the human, rat or mouse H1t genes are located near testicularly expressed H4 genes which code for the same H4 amino acid sequences as other H4 genes from the same species.

Organization of mammalian histone gene clusters

The majority of histone genes in the murine and human genomes is clustered at specific chromosomal sites. Except the H1° gene, all known human H1 genes and surrounding core histone genes are located on chromosome 6 [3]. A minor portion of core histone genes maps to chromosome 1 [46], and the solitary H1° gene is located on the long arm of chromosome 22 [3]. The situation in the murine genome appears to be similar, since a major histone gene cluster including the H1t gene has been mapped to chromosome 13 [26, 75], and the murine H1° gene is on chromosome 15 in a region which is syntenic with the region on the human chromosome 22, where the human H1° gene is located [3, 11].

The human H1t gene, which is expressed in pachytene spermatocytes (see below) forms part of the major gene cluster on chromosome 6, which also contains the other H1 genes [3]. Thus, the generation of H1 histone patterns, which are characteristic for cells of specific stages of spermatogenesis, must depend on a differential regulation of the genes within that major cluster. In addition, the expression of the H1° gene, which appears to be developmentally regulated

Source : MNHN, Paris

during differentiation of several cell types [99], must undergo tissue-specific control. It is preferentially expressed during early stages of spermatogenesis [42] and in somatic cells it mainly appears upon terminal differentiation [99].

The genes coding for the testicularly expressed histones TH2A, TH2B and TH3 have not yet been mapped to specific chromosomal sites. They also may form part of the major histone gene cluster. GRIMES *et al.* [49] have shown that an H4 gene, which is located near the rat H1t gene, is testicularly expressed. It has the same primary structure as other mammalian H4 proteins. On the basis of its variant nucleotide sequence and testicular expression, this gene may be termed H4t [49, 94]. Its association with the H1t gene, which is located within the major cluster of somatically expressed histone genes, implies that it is not a solitary gene. In contrast to the H1t gene, expression of this neighbouring H4 (H4t) gene is not confined to spermatogenic cells, but its mRNA also has been detected in a rat myeloma cell line.

Cell type specific histone patterns at different stages of spermatogenesis

The H1 patterns of different somatic cell types or germ cells are not uniform, but vary in their H1 subtype composition. In several mammalian species, five main type H1 protein species (termed H1a-H1e) were described [62]. In rat testes, the subtypes H1a-H1e can be detected (H1a and H1c predominating) during all stages of spermatogenesis until the primary spermatocyte stage [12, 62]. Similarly, the subtypes H1a and H1c predominate in mouse germ cells until the meiotic prophase [62]. Immunocytochemical analysis of murine tubuli seminiferi showed the greatest level of reactivity in primary spermatocyte nuclei using antibodies against H1a [79]. Developmental studies showed that the first expression of the H1a gene occurs in 7 day old mice at a stage when intermediate and B type spermatogonia appear [79]. *In situ* hybridizations with human testis detected the mRNA coding for human H1.1 (equals H1a according to [76]) until the stage of round spermatids [14, 15]. Thus, the subtype H1a appears to be a major constituent in the chromatin of mammalian germ cells [13, 67]. In addition, the subtypes H1b, c and d contribute to the germ cell chromatin [62, 67, 78].

The H1 subtype H1° has been described in unfractionated mammalian testis cell preparations [83, 85, 86]. The predominant expression of the H1° gene in spermatogonia was suggested by promoter studies of GARCIA-IGLESIAS *et al.* [42]. In that work, the H1° promoter was ligated upstream of a ß-galactosidase gene and the expression of this construct was monitored in transgenic mice. The analysis showed that the promoter was used in several tissues, such as specific cell types in kidney, brain and testis. Testicular mRNA synthesis was mostly confined to spermatogonia, but immunofluorescence studies with H1° antibodies indicated expression in Sertoli cells, too. Thus, expression of the H1° gene may be confined to early stages of spermatogenesis, but somatic cells in the testis also express the H1° gene.

The H1t protein is absent from spermatogonia and is first detected in pachytene spermatocytes [35, 60, 69]. This has been demonstrated at the protein level in chromatin from mouse and rat cells fractionated by elutriation centrifugation [44]. After the cloning of the genes coding for the human, murine and rat H1t proteins [22, 34, 35], Northern blot and *in situ* hybridization analysis has confirmed these protein data showing that the mRNA is only found in pachytene spermatocytes [60] whereas the proteins are preserved in the subsequent stages until histone replacement by transition proteins [69].

The major change in chromatin structure during the meiotic prophase is also evident in the H2A/H2B class of histones. The subtypes TH2A and TH2B both become first detectable in pachytene spermatocytes of rat and mouse [20, 67, 78, 92]. The subtype H2A.X, which, like H1°, is a non replication-dependent histone [72, 96], has been detected in type A spermatogonia [64]. Expression of a modified H2B protein has been found during mouse spermiogenesis [70]. In a cDNA library constructed from spermatid RNA, an H2B cDNA sequence was observed which was extremely similar to other mouse H2B gene sequences, but the C-terminus coded for

Source : MNHN, Paris

12 additional amino acids, 7 of which were hydrophobic. Northern blots with RNA from other tissues indicated that this transcript was testis-specific [70]. Similarly, a polyadenylated H2A mRNA was detected in mouse round spermatids [71].

The histone to protamine transition during human spermiogenesis does not result in a complete removal of all histones [43, 52]. About 15% of the human sperm DNA appears to remain associated with core histones, i.e. mainly with H2A (H2A.X and trace amounts of H2A.Z), several H2B isotypes, H3.1, H3.3 and highly acetylated H4 [43]. In contrast to these remaining core histones, no association of any H1 subtype with mature sperm chromatin has been observed.

The gradual changes of the core and linker histone moieties during the development of male germ cells and the changes in chromatin morphology and gene activity suggest a functional role for the individual histone subtypes. However, correlations between specific structural features of histones and functional differences have not yet been established in any somatic or germ cell system. H1°, which appears to be confined to early stages of spermatogenesis, is correlated with terminal differentiation in several cell types (for review, see [99]). Its avian countertpart H5 is confined to the condensed, transcriptionally inactive nuclei of avian red blood cells [5]. The high arginine content [30, 31] of the H5 histone is considered as one of the reasons for its condensing capacity. Compared with the other H1 subtypes, H1t is also enriched in arginine, but DE LUCIA *et al.* [29] have shown by circular dichroism analysis that H1t has a lower condensing capacity than the other H1 subtypes. Thus, H1t may even contribute to activating effects in the chromatin of developing germ cells rather than repressing nuclear activity. It may thus help to decondense the chromatin structure for the specific needs of the meiotic and haploid stages of germ cell development.

Postsynthetic histone modifications

Posttranslational modifications of histone proteins have been primarily observed at spermatid stages of spermatogenic cell development. Recently, the phosphorylation of H1t in elongating spermatids has been described [69]. In the same study, which used vitamin A as a means to synchronize rat seminiferous epithelia into few stages of spermiogenesis, additional bands of H2A.1, H2A.2 and TH2A were observed and were interpreted as postsynthetic modifications. A complex pattern of phosphorylation of the H2A.X subtype has been observed in murine testicular cells [45]. Another type of histone modification is the conjugation with ubiquitin. This has been described for H2A histones during rooster spermatogenesis [1].

The most impressive modification of histones during spermatogenesis is their hyperacetylation. This modification of the H4 histone structure is correlated with a broad spectrum of cellular processes, including transcriptional control and chromatin assembly (for review, see [95]). H4 hyperacetylation occurs in elongating spermatids [47, 48, 68]. This is the stage when displacement of histones by transition proteins begins [69, 73]. Thus, the association of highly acetylated H4 with the stage of histone displacement in rat spermatids is in agreement with the idea that reducing the positive charge of specific lysine residues may help to displace histones from chromatin during spermiogenesis.

Regulation of testicular histone gene expression

The location of spermatogenesis related histone genes within clusters of somatic histone subtype genes implies that control steps discriminate between the different member genes of the gene cluster. This control may take place at the transcriptional level, but also posttranscriptionally, i.e. during processing of the primary transcript or by influencing the stability of specific histone mRNAs (for review, see [74]). At the transcriptional level, promoter structures of specific histone genes may contain sequence motifs where interaction with germ cell-specific transcription factors controls the specific expression of the respective genes.

Source : MNHN, Paris

The mechanism of H1 histone gene regulation in *somatic* cells is not yet fully understood. Sequence analysis of H1 gene promoters in all vertebrate systems studied revealed that the heptanucleotide AAACACA is conserved at a position 100 nucleotides upstream of the transcription start site [25, 27, 28]. Functional studies indicated the involvement of this H1-box in the S-phase-dependent expression of H1 genes [27, 28, 61], but variants of this sequence motif have been observed [38]. A second sequence element, which is involved in the regulation of H1 genes, is the CCAAT box, which is the binding site for an H1-specific regulatory factor [41]. The sequence analysis of the rat, human and murine H1t promoters revealed that their sequences contained all main features of S-phase-dependent H1 genes: TATA-box, GC-rich element, CCAAT-motif and H1-box [22, 34, 35, 50, 51]. Thus, the known regulatory elements within H1t promoter structures apparently do not reflect the fact that the H1t gene is not transcribed during DNA replication, but at the pachytene stage of the meiotic prophase. GRIMES and coworkers [50, 51] searched for a testis-specific element and defined the palindromic hexanucleotide CCTAGG, which is located between the GC-rich element and the CCAAT-box of the rat H1t promoter as the testis-specific promoter element [50, 51]. This element was identified as the site of interaction of testis-specific DNA-binding proteins at the promoter in pachytene spermatocytes [51]. Further support for a functional role of this sequence element may be derived from the human H1t promoter, where this sequence motif is conserved at the same site [32, 34]. The palindromic arrangement, however, may not be mandatory, since the mouse H1t promoter shows a varied element, CCTGGG, at the same location [35].

The rat TH2A and TH2B genes are grouped together, and they are divergently transcribed from a joint promoter region of about 240 nucleotides in between the two genes [56-58]. In both directions, TATA- and CCAAT-boxes are located upstream of the two genes. In addition, the TH2B gene promoter contains the OctI element ATTTGCAT, which is a characteristic regulatory element in all H2B gene promoters [40] but also in control regions of several other genes. For example, variant Oct factors binding to such elements have been detected during mouse embryogenesis and are specifically expressed in germline cells [81]. In conclusion, the promoter arrangement of the TH2B gene does not vary from consensus H2B promoter structures and it does not reflect the replication-independent, testis-specific expression of this histone gene. Functional studies with the TH2B promoter in fibroblast cells revealed that the CCAAT- and octamer elements of this promoter are involved in the S-phase dependent expression of the TH2B gene when transfected into these *somatic* cells [56-58]. Subsequent studies showed that differential methylation at specific sites of the TH2B promoter contributes to the tissue-specific transcription of this TH2B gene [20] and that a repressor protein specific for the rat TH2B gene was present during early stages of spermatogenesis [63].

The gene coding for TH3, which has been described as a testis-specific H3 subtype in rat spermatogonia [91], has not yet been detected. Thus, no data on cell specific regulation of testicular H3 histones exist. As mentioned above, GRIMES *et al.* [49] have shown that an H4 gene is closely associated with the rat H1t gene. S1 nuclease analysis has shown that this particular H4 gene is transcribed in the testis predominantly during the pachytene stage, but it is also expressed in a tumor cell line. This is in contrast to the neighbouring H1t gene, which is solely transcribed in pachytene spermatocytes [50, 94].

The control of histone gene expression is not restricted to transcriptional regulation (for review, see [74]). Processing of the primary transcript and mRNA stability of replication dependent histone gene products depend on the presence of a dyad symmetry element at the 3' end of the mRNA, which is not polyadenylated [8, 82]. The only exceptions from this rule are the S phase-independent replacement histone variants, such as H1°, H3.3, H2A.Z or H2A.X, which are all encoded by polyadenylated histone mRNAs. Remarkably, the H1t genes of rat, mouse and man show the same dyad symmetry elements as replication dependent histone genes and the mRNAs are non polyadenylated.

Source : MNHN, Paris

Posttranscriptional regulation of testicular histone gene expression

As mentioned above, main type, S phase-dependent histone mRNAs in somatic cells are poly(A)$^-$ in contrast to the mRNAs encoding the replacement histone subtype mRNAs. In addition to these specific subtype mRNAs, polyadenylated testicular histone mRNAs have been described [39]. These are at least in part derived from genes which are transcribed to poly(A)$^-$-mRNA in somatic cells. A poly(A)+ histone H2B mRNA with an extended reading frame and a consensus AAUAAA polyadenylation signal has been detected in mouse spermatids [70]. Recently, a polyadenylated H2A gene transcript was found in murine round spermatids. In this case, the poly(A) tail was not preceded by the somatic AAUAAA signal sequence [71]. In a detailed analysis of histone mRNAs in chicken spermatids, CHALLONER *et al.* [19] detected an H2B mRNA subpopulation, which was polyadenylated despite the fact that the histone mRNA was derived from a gene which is transcribed as a poly(A)$^-$-mRNA in somatic cells. The transcript from this same gene was elongated by 26 or 28 nucleotides beyond the histone mRNA consensus termination site, and a poly(A) tail was added to this elongated mRNA.

A major step in histone gene regulation is the control of mRNA degradation [74]. This has not been specifically studied in testicular histone gene expression, but the addition of poly(A) tails to part of the histone mRNA population during spermatogenesis suggests that it is a means to increase the stability of this mRNA, which is either synthesized at post-meiotic stages or is synthesized at early spermatogenesis and is preserved for later stages of development, when a certain pool of mRNAs for histone replacement may be needed.

Conclusions

Modulation of the chromatin structure during spermatogenesis requires changed patterns of histone proteins and histone modifications which contribute to restructuring of chromatin and to the transition towards the inactivation of the genome in generating the condensed genome of mature sperm. Specific histone subtypes, which differ from their somatic counterparts have been described for all histone classes except H4. The most drastic changes in histone gene expression and chromatin restructuring occur during the meiotic prophase, when specific subtypes of H1, H2A and H2B, i.e. H1t, TH2A and TH2B, are synthesized. These testis specific isoforms remain associated with the chromatin of cells during the haploid stages of sperm cell differentiation. At this spermiogenesis period, remodelling of chromatin before the final deposition of protamines may require a specific chromatin structure which is accessible for regulatory factors such as non-histone proteins and for transition proteins replacing the histone moiety. This specific chromatin structure may be established by specific subsets of core and linker histones and by their posttranslational modification.

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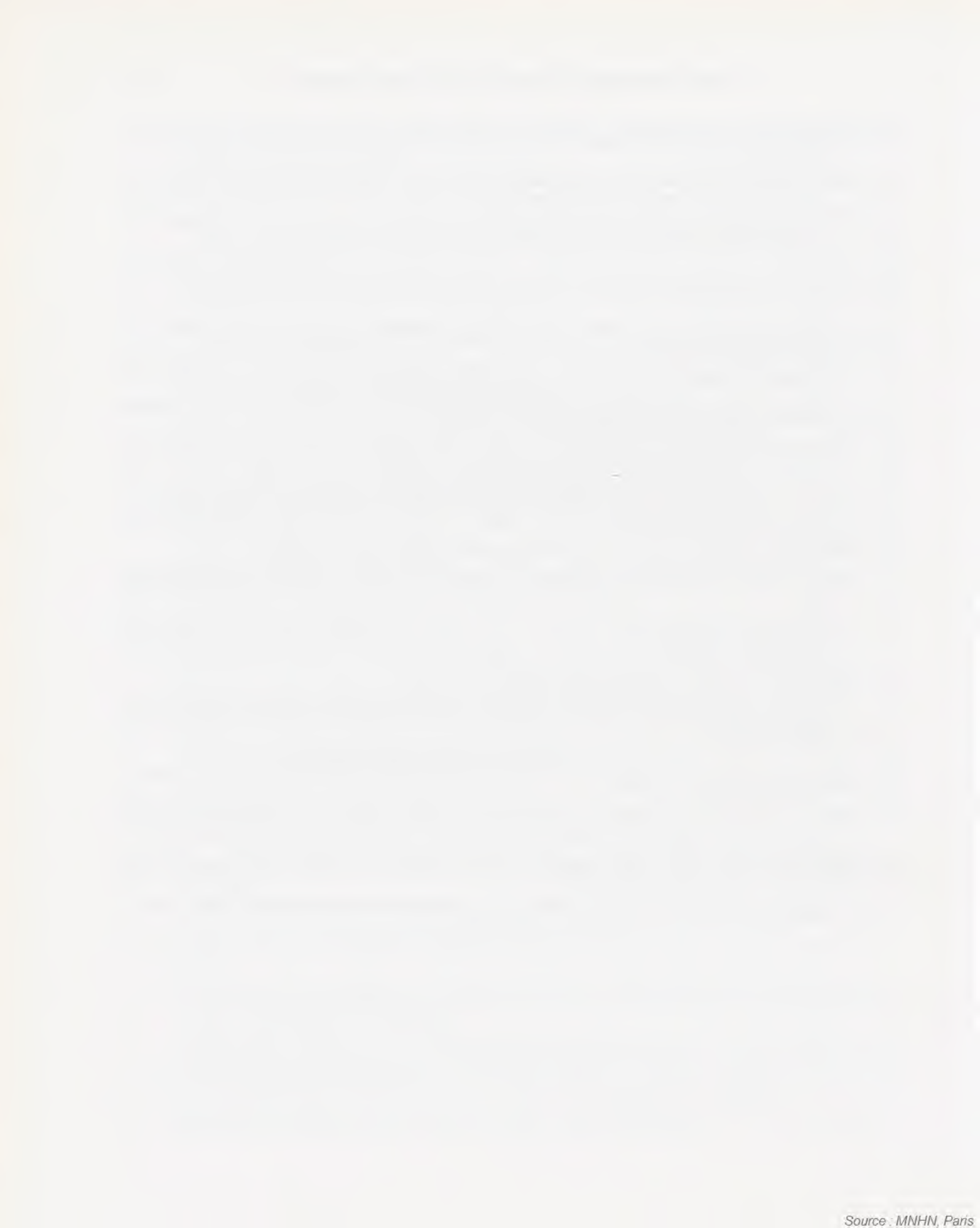

Source : MNHN, Paris

Sequence, Evolution and Transcriptional Regulation of Avian-Mammalian P1 Type Protamines

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ABSTRACT

Methodological approaches to protamine P1 sequence determination have evolved from the initial protein sequencing methods to the robust cloning and PCR-based techniques. Twenty-seven different mammalian-avian P1 type protamine genes and 32 different P1 amino-acid sequences are now available and allow detailed phylogenetic analysis and the study of transcriptional control mechanisms. All mammalian-avian P1 type protamines contain a well conserved N-terminus with the consensus "ARYR" followed by alternating "S/T-S-S" phosphorylatable residues. Eutherian mammalian P1s contain cysteine residues, whereas birds, prototherian and metatherian protamines lack cysteine. Thus cysteine appeared after the divergence of marsupials, monotremes and placental lineages. Overall detailed phylogenetic analysis of the gene sequences indicates that the evolution of P1 genes is in agreement with the expected species evolution supporting that these genes have evolved vertically.

RÉSUMÉ

Séquence, évolution et régulation transcriptionnelle des protamines de type P1 des Oiseaux et Mammifères

Les approches méthodologiques de détermination de séquence des protamines P1 ont évolué depuis les premières méthodes de séquençage de protéines jusqu'aux techniques fiables basées sur le clonage et la réaction d'amplification en chaîne. Vingt-sept gènes différents de protamines de type P1 des Oiseaux et Mammifères et trente-deux séquences différentes d'acides aminés sont maintenant disponibles et permettent une analyse phylogénique et une étude des mécanisme de contrôle de la transcription. Toutes les protamines de type P1 des Oiseaux et Mammifères contiennent une extrémité N-terminale bien conservée avec la séquence consensus "ARYR" suivie par la séquence alternée de résidus phosphorylables "S/T-S-S". Les protamines P1 des Mammifères Euthériens contiennent des résidus de cystéine, alors que les Oiseaux, les Prothériens et les Méthathériens n'en ont pas. La cystéine est donc apparue après la divergence des lignées des Marsupiaux, des Monotrèmes et des Placentaires. Une analyse phylogénique générale et détaillée des séquences de gènes indique que l'évolution des gènes des P1 est en accord avec l'évolution attendue des espèces, ce qui indique que ces gènes ont évolué verticalement.

Protamines are small (30-60 amino acids) and very positively charged proteins (40-70% arginine) which appear at the late stages of spermatogenesis in many but not all animal, and some plant, species [6, 11, 16, 21-23, 25, 43, 50, 52, 60, 69-73]. In those species in which they occur, such as in all mammals [6, 52], birds [13, 52, 53,], some teleost fish [11, 16], some reptiles [25, 70] and amphibians [25] they replace most of the histones during spermiogenesis and

OLIVA, R., 1995. — Sequence, evolution and transcriptional regulation of avian-mammalian P1 type protamines. *In*: JAMIESON, B. G. M., AUSIO, J., & JUSTINE, J.-L. (eds), Advances in Spermatozoal Phylogeny and Taxonomy. *Mém. Mus. natn. Hist. nat.*, **166** : 537-548. Paris ISBN : 2-85653-225-X.

Source : MNHN, Paris

become the major sperm nuclear protein [47, 48, 52, 54]. There are two basic groups of protamines in mammals; the P1 protamines which have been found in all mammalian species that have been analyzed and the P2 protamines which have been found in humans [5, 8, 42, 79, 80] and a limited number of other mammals such as mouse, guinea pig and stallion [10, 59, 66]. However, pro-P2 protamine genes have been sequenced from eight species of primates [66]. Both types of protamines contain cysteine which can form disulphide bonds and contribute to the stability of the condensed sperm nucleus. Bird protamines lack cysteine [13, 45, 49, 52] although they are clearly related to mammalian P1 protamines as several identical amino acid sequence motifs are present in both cases. Because of the high variability of protamines and protamine genes it is very difficult at present to explain the evolution of these proteins within an entire phylum. In many cases the limited number of sequences available precludes their connection into a coherent evolutive pathway. Thus the focus in this review has been placed in the avian mammalian-mammalian P1 type protamine for which a considerable amount of information is now available. Other papers and reviews cover other vertebrate or invertebrate groups ([4, 12, 13, 16, 25, 43, 49, 52, 60, 70, 71, 73], see also CHIVA, SAPERAS, CACERES & AUSIO, this volume, and PRATS & CORNUDELLA, this volume). Protamine genes are a clear example of highly tissue-specific genes. However the mechanisms that direct their specific expression in the testis are not fully understood [18, 22, 50, 52, 80]. Thus the last section of this review covers the progress made in the understanding of the transcriptional control of the P1 genes.

RESULTS AND DISCUSSION

Methodological approaches to protamine P1 sequence determination:

The methods initially available to sequence protamines were based on end group analysis, proteolytic digestion, isolation, sequencing, and overlapping of the protamine peptides. The presence of several arginine tracts in each protamine with very similar sequences made this approach technically difficult. The first reported avian-mammalian P1 type complete sequences using these methods corresponded to bull [15; Table 1], *Gallus domesticus* [45] and boar [76]. Some discrepancies in the initial reported sequences were found when the corresponding protamines were re-examined by automated micro-sequencing or by cloning of the protamine cDNAs and genes [37, 49, 40]. Subsequently the use of automated protein micro-sequencing led to the determination of the sequences of human P1 [41], stallion P1 [2, 9], ram [71] and rabbit, goat and rat [3]. Partial sequences corresponding to a few N-terminal residues have also been reported for many mammalian protamines [6] (Table 1).

Simultaneously to the onset of the use of automated micro-sequencing, the methods of the cDNA synthesis, clonning and sequencing were also developed and applied to protamine genes (Table 1). The first mammalian protamine cDNA sequence corresponded to mouse P1 [29]. Since no probes were initially available to screen the cDNA library, this initial sequence was obtained by characterization of selected clones preferentially expressed in spermatids [28]. Subsequently, the use of the mouse P1 cDNA clone as a probe led to the determination of the sequence of bovine protamine P1 cDNA [35]. Simultaneously, the bovine protamine P1 cDNA sequence was also independently obtained using oligonucleotides designed from the previously known amino acid sequence [31]. The mouse P1 cDNA also led to the isolation of the boar protamine 1 cDNA [37] and rat P1 cDNA [30]. The bovine probe led to the isolation and sequencing of the human protamine P1 cDNA [36]. However the mammalian probes would not recognize the avian protamine genes because of marked divergence in the nucleotide sequences between these species. Thus the cDNA sequence corresponding to rooster protamine was obtained by random sequencing of 210 clones from a rooster testis cDNA library until the sequence of one clone predicted an amino acid sequence similar to the previously reported at the protein level for galline [55]. The availability of a cDNA probe from galline led to the rapid isolation and sequencing of

Source : MNHN, Paris

TABLE 1. — Chronological list of reports on protamine P1 sequences with indication of the species and methods used for sequencing.

Year	Protamine sequence/s reported	Method	Reference
1972	Bull (*Bos taurus*)	Protein sequencing	[15]
1976	Galline (*Gallus domesticus*)	Protein sequencing	[45]
1976	Rat -partial-(*Rattus norvegicus*)	Protein sequencing	[27]
1983	Boar (*Sus scrofa*)	Protein sequencing	[76]
1984	Ram (*Ovis aries*)	Protein sequencing	[71]
1985	Human (*Homo sapiens*)	Protein sequencing	[41]
1985	Mouse (*Mus musculus*)	cDNA	[29]
1986	Bull (*Bos taurus*) (corrected)	Protein sequencing	[31]
1987	Bull (*Bos taurus*)	cDNA	[31]
1987	Bull (Bos *taurus*)	cDNA	[35]
1987	Stallion (*Equus caballus*)	Protein sequencing	[9]
1987	Stallion (*Equus caballus*)	Protein sequencing	[2]
1987	Human (*Homo sapiens*)	cDNA	[36]
1988	Galline (*Gallus domesticus*)	cDNA	[55]
1988	Bull (*Bos taurus*)	Genomic phage library	[32]
1988	Mouse (*Mus musculus*)	Genomic phage library	[24]
1988	Mouse (*Mus musculus*)	Protein sequencing	[10]
1988	Rabbit (*Oryctolagus cuniculus*)	Protein sequencing	[3]
1988	Goat (*Capra hircus*)	Protein sequencing	[3]
1988	Rat (*Rattus norvegicus*)	Protein sequencing	[3]
1988	Boar (*Sus scrofa*)	cDNA	[37]
1989	Chicken (*Gallus domesticus*)	Genomic cosmid library	[49]
1989	Quail (*Coturnix japonica*)	cDNA	[53]
1989	Rat (*Rattus norvegicus*)	cDNA	[30]
1989	Wallaby (*Cricetulus migratorius*)	Protein sequencing	[7]
1989	Dwarf Hamster (*Phodopus sungorus*)	Protein sequencing	[6]
1989	Rhesus monkey -partial-(*Macaca mulatta*)	Protein sequencing	[6]
1990	Human (*Homo sapiens*)	Genomic cosmid library	[18]
1991	Marmoset (*Saguinus imperator*)	PCR with genomic DNA	[63]
1992	Boar (*Sus scrofa*)	Genomic phage library	[26]
1993	Whale (*Orcinus orca*)	PCR	[1]
1993	Human (Mediterranean, Sudanese, Korean, American Indian)	PCR	[62]
1993	Rat -5´region-(*Rattus norvegicus*)	PCR	[64]
1993	Guinea pig -5´region-(*Cavia porcellus*)	PCR	[64]
1993	Gorilla -5´region-(*Gorilla gorilla*)	PCR	[64]
1993	Orangutan -5´region-(*Pongo pygmaeus*)	PCR	[64]
1993	Anubis baboon -5´region-(*Papio doguera*)	PCR	[64]
1993	Red monkey -5´region- (*Cercopithecus patas*)	PCR	[64]
1993	Opossum (*Didelphis marsupialis*)	PCR	[78]
1993	Common chimpanzee (*Pan troglodites*)	PCR	[68]
1993	Pygmy chimpanzee (*Pan paniscus*)	PCR	[68]
1993	Gorilla (*Gorilla gorilla*)	PCR	[68]
1993	Orangutan (*Pongo pygmaeus*)	PCR	[68]
1993	Gibbon (*Hylobates lar*)	PCR	[68]
1993	Red monkey (*Cercopithecus patas*)	PCR	[68]
1993	Marmoset (*Saguinus imperator*)	PCR	[68]
1993	Red howler (*Alouatta seniculus*)	PCR	[68]
1993	Platypus (*Ornithorincus anatinus*)	PCR	[67]
1993	Echidna (*Tachyglossus aculeatus*)	PCR	[67]
1994	Rat (*Rattus norvegicus*)	PCR	[61]
1994	Guinea pig (*Cavia porcellus*)	PCR	[61]
1994	Cat (*Felis catus*)	PCR	[61]
1994	Bear (*Ursus americanus*)	PCR	[61]
1994	Elephant (*Elephas*)	PCR	[61]
1994	Horse (*Equus caballus*)	PCR	[61]
1994	Camel (*Camelus dromedarius*)	PCR	[61]
1994	Deer (*Odocoideus virginianus*)	PCR	[61]
1994	Elk (*Cervus elaphus*)	PCR	[61]
1994	Moose (*Alces alces*)	PCR	[61]
1994	Gazelle (*Gazella dorca*)	PCR	[61]

Source : MNHN, Paris

another avian protamine, the quail (*Coturnix japonica*) [53], as well as to the isolation and sequencing of the chicken genomic clones [49]. The same approach using cDNA as probes to screen genomic libraries was also followed to obtain the genomic sequences corresponding to bull P1 [32], mouse P1 [24], human P1 [18] and boar P1 [26] (Table 1). The availability of probes for all these genes also led to the determination of their copy number which proved to be one copy per haploid genome for P1 protamines in mammals, two identical genes (coding region) per haploid genome in *Gallus domesticus*, and one copy of P2 per haploid genome in mammals. This indicated that the numerous basic protamine type molecules present in the sperm nucleus [P1, P2, P3, P4 and others] of mammals corresponded only to two types of protamines [P1 and P2]. The P1 genes are located adjacent to other spermatid-specific genes in the mammalian genome [19, 34, 46]. As some mammalian protamine genes were sequenced by independent laboratories, some minor discrepancies in the reported sequences also emerged, such as in the bull genes [31, 35] and between the human P1 sequence initially reported [18] with that subsequently redetermined in several independent individuals [62].

The availability of the P1 genomic sequences from human, bull, mouse and boar allowed their comparison in a search for conserved flanking sequences from which to design consensus oligonucleotides. This approach proved to be extremely efficient leading to the isolation and sequencing of the *Saguinus imperator* P1 protamine [63], *Orcinus orca* P1 [1], and subsequently several primates (common chimpanzee, pygmy chimpanzee, gorilla, orangutan, gibbon, *Cercopithecus patas*, *Alouatta seniculus*) [68], several human individuals (Mediterranean, Korean, Sudanese, American Indian) [62], additional eutherian mammals (rat, guinea pig, cat bear, elephant, horse, camel, deer, elk moose, gazelle) [61] and the monotremes, platypus and echidna [67]. The determination of a Wallaby partial amino acid sequence [7] by protein micro-sequencing led to the sequencing of the opossum protamine P1 [77, 78]. A similar PCR-based approach was followed to amplify and sequence the promoter region of the rat, guinea pig, gorilla, orangutan, anubis baboon and red monkey [64].

Although PCR from genomic DNA is a very valuable tool in the amplification and sequencing of new protamine genes it does not provide information on whether the sequenced genes are expressed or not (for instance, if a sequenced gene is a pseudogene). In the case of P1 genes, the fact that all mammalian species where the sperm nuclear protein content has been analyzed contain protamine P1 together with the single copy number of the P1 genes in mammals, suggests that most (if not all) of the mammalian species whose P1 sequence has been determined by PCR also express the sequenced gene. However this could be a limitation in the prediction of functional properties based on the derived amino acid sequence in the case of those proteins (such as P2 protamine) which are not ubiquitously expressed in mammals [66].

The availability, at present, of a large number of P1 sequences should allow design of new primers with which to amplify and sequence the P1 genes corresponding to species which have remained so far elusive. In the case of the P1 genes the PCR approach has been successful in the amplification of sequences corresponding to members of the class from which the oligos were predicted (e.g. mammals), but failed in the amplification of the P1 genes corresponding to other classes (e.g. reptiles or amphibians). Thus determination of the sequences of protamine genes in other vertebrate classes (or in other phyla) will probably require laborious groundwork based on either protein micro-sequencing (followed by oligonucleotide design and PCR) or cDNA based approaches [29, 55]. However once one or a few nucleotide sequences became available in the additional phyla and classes [4, see PRATS & CORNUDELLA, this volume], the same PCR-based approach successfully used in mammalian-avian P1s should also work in the determination of protamine sequences corresponding to most of the members of other classes.

Source : MNHN, Paris

```
                10        20        40        40        50        60
Bull            ARYRCCLTH--SGSRCRRRRRRRCRRRRRR-FG------RRRRRR---VCCRR---YTVIRCTRQ
Goat            ARYRCCLTH--SRSRCRRRRRRRCRRRRRR-FG------RRRRRR---VCCRR---YTVVRCTRQ
Ram             ARYRCCLTH--SRSRCRRRRRRRCRRRRRR-FG------RRRRRR---VCCRR---YTVVRCTRQ
Orca            ARNRC-RSP--SQSRCRRPRRR-CRR-RIR-CC------RRQ-RR---VCCRR---YTTTRCARQ
Boar            ARYRCCRSH--SRSRCRPRRRR-CRRRRRR-CC------PRR-RRA--VCCRR---YTVIRCRRC
Horse           ARYRCCRSQ--SQSRCRRRRRRRCRRRRRR-SV------R-Q-RR---VSCRR---YTVLRCRRRR
Mouse           ARYRCCRSK--SRSRCRRRRR-RCRRRRRR-CC------R-RRRR---RCCRRRRSYT-IRCKKY
Rat             ARYRCCRSK--SRSRCRRRRR-RCRRRRRR-CC------R-RRRR---RCCRRRRSYT-FRCKRY
Rabbit          VRYRCCRSQ--SRSRCRRRRR-RCRRRRRR-CC------QRRRVR---KCCRR--TYT-LRCRRY
Pygmy chimp     ARYRCCRSQ--SRSRCYRQRR--SRRRKRQ-SC------QTQRRAM--RCCRRR--SR-LRRRRH
Common chimp    ARYRCCRSQ--SRSRCYRQRQ-RSRRRKRQ-SC------QTQRRAM--RCCRRR--SR-MRRRRH
Gorilla         ARYRCCRSQ--SRSRCYRQRQ-TSRRRRRR-SC------QTQRRAM--RCCRRR--NR-LRRRKH
Human           ARYRCCRSQ--SRSRYYRQRQ-RSRRRRRR-SC------QTRRRAM--RCCRPR--YR-PRCRRH
Orangutan       ARYRCCRSQ--SQSRCCRRRQ-RCHRRRRR-CC------QTRRRAM--RCCRRR--YR-LRCRRH
Red monkey      ARYRCCRSQ--SRSRCCRQRR-RCRRRRRR-RC------RARRRAM--KCCRRR--YR-LRCRRY
Gibbon          ARYRCCRSQ--SRSRCYRRGQ-RSRRRRRR-SC------QTRRRAM--RCCRPR--YR-LRRRRH
Red howler      ARYRCCRSRSLSRSRCYRQRP-RCRRRRRR-SC------RRP-RAS--RCCRRR--YR-LRRRRY
Marmoset        ARYRCCRSQ--SRSRCYRQRR-RGRRRRRR-TC------RRR-RAS--RCCRRR--YK-LTCRRY
Opossum         ARYRR-RSRSRSRSRYGRRRRR--SRSRRRR-SRRRRRRRG---RRG--RGYHRRSPHRRRRRRRRR
Echidna         ARFRPSRSR--SRSLYRRRRR--SRRQRSRRGGRQTGPRKITRRGRGRGKSRRRRGRRSMRSSRRRRRRRRN
Platypus        ARFRRSRSR--SRSLYRRRRR--SRR-----GGRQTRSRKLSR-SRRRGRSRRRKGRRSRRSSRRS--RRRN
Chicken         ARYRRSRTR--SRSPRSRRRRRRSGRRR---SPRRRRRYGSARRSRRSVGGRRRR-YGSRRRRRRRY
Quail           ARYRRTRTR--SRSR----RRRRSRRRR---SSRR-RRYGRSRRSYRSVGRRRRR-YGRRRRRRRY
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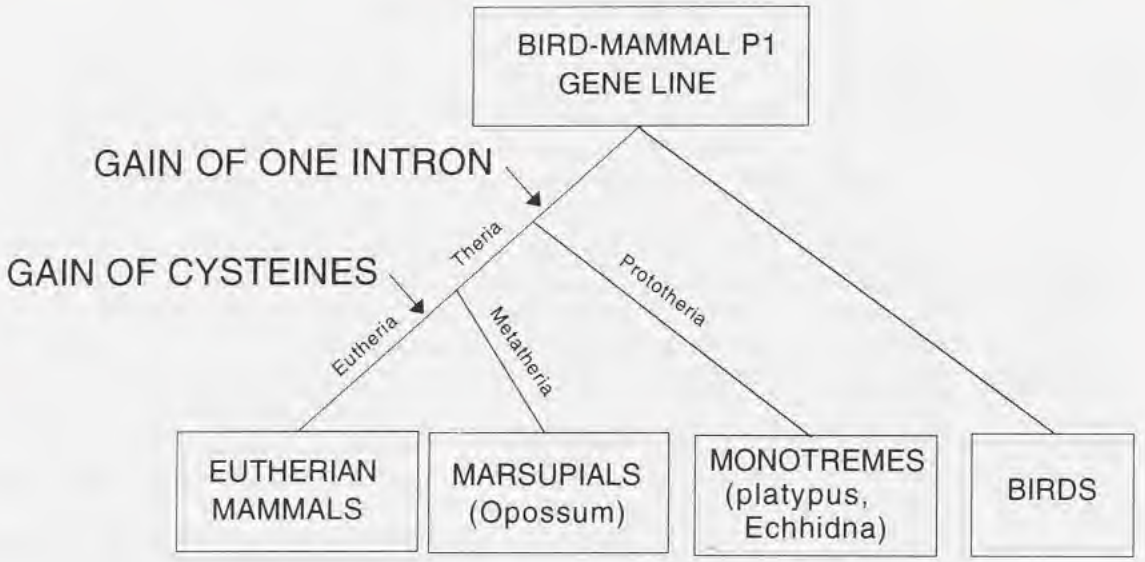
FIG. 1. — Alignment of the reported avian-mammalian P1 amino acid sequences (see Table 1).

Implications for protamine P1 gene evolution

The coincidence of the four C-terminal amino acids between mammalian P1s and galline (the protamine from *Gallus domesticus*) has been known for nearly two decades [45]. However, because of the lack of cysteine residues in bird protamines and the presence of these amino acids in mammalian protamines, both types of protamines had been classically classified as belonging to different types. According to BLOCH's classification, galline was type 1 (or a true protamine) whereas mammalian protamines were type 2 (stable or keratinous protamines). The similarity between mammalian and rooster protamine became stronger when the sequence predicted from the genome (and in accordance with the re-sequence obtained for the N-terminus of the protein) [49]; (Fig. 1) was used instead of the initial sequence reported [45]. For instance, the single threonine residue present in galline occupies exactly the same position as that present in ram and bull [52] (Fig. 1). The determination of the quail protamine sequence [53] also revealed the presence of the N-terminal ARYR motif and a size (56 residues) closer to mammalian P1s (50 residues) than those with galline (61 residues; Fig. 1). An alternating triple phosphorylatable site (T/S-S-S) is also found at positions 9-15 in all avian-mammalian P1 protamines (Figs 1, 2) [6, 20, 52, 57, 58]. The size among bird protamines appears consistently similar to that of mammals (Fig. 1) [13, 14]. Altogether the data strongly suggest the existence of an avian-mammalian protamine gene line during evolution (Fig. 2).

A new insight has come form the recent determination of the sequence corresponding to the prototherian (the monotremes platypus and echidna) [67] and the metatherian protamines (the marsupials) [7, 69, 77, 78]. All these sequences lack cysteine and the corresponding genes contain one intron. Thus these species are closer to birds according to their lack of cysteine but closer to eutherian mammals according to the presence of the single intron. Detailed phylogenetic analysis indicates that these sequences are half way between eutherian mammals and birds [7, 49, 67, 77, 78]. Based on the limited (but significant) similarity in the introns between prototherian

Source : MNHN, Paris



N-TERMINUS	ARYR	ARYR	ARFR	ARYR
T/S - S - S (9-15)	YES	YES	YES	YES
INTRON	YES	YES	YES	NO
CYSTEINES	YES	NO	NO	NO

FIG. 2. — Possible pathway of mammalian-avian protamine P1 gene evolution.

and eutherian mammals it was concluded that the introns in the protamine P1 genes of monotremes, marsupials and eutherian mammals were derived from a single intron that was probably inserted into the ancestral gene prior to the divergence of the theria and prototheria 150 to 170 million years ago [67] (Fig. 2).

The comparison of all protein and DNA sequences further strengthens the idea that protamines are amongst the most rapidly diverging proteins studied [68]. This variation may allow discrimination of closely related species or even individuals in some cases. For instance, a sequence polymorphism has been found in the human P1 gene [62]. Molecular analysis of the P1 genes from nine primates revealed that within primates the rate of evolutionary change is much higher than that within other mammalian orders [68]. Interestingly, the primate P1 data confirm that human-gorilla-chimpanzee P1 protamines are indeed very similar but, unlike the slightly closer association between chimpanzee and human derived from analysis of other genes [66], the human-gorilla relationship is slightly favoured in the case of the P1 genes [68].

Overall phylogenetic analysis of all P1 sequences (Table 1) indicates that the molecular evolution of P1 genes is in agreement with the expected species evolution supporting that these genes have evolved vertically [61, 83] (Fig. 2).

Source : MNHN, Paris

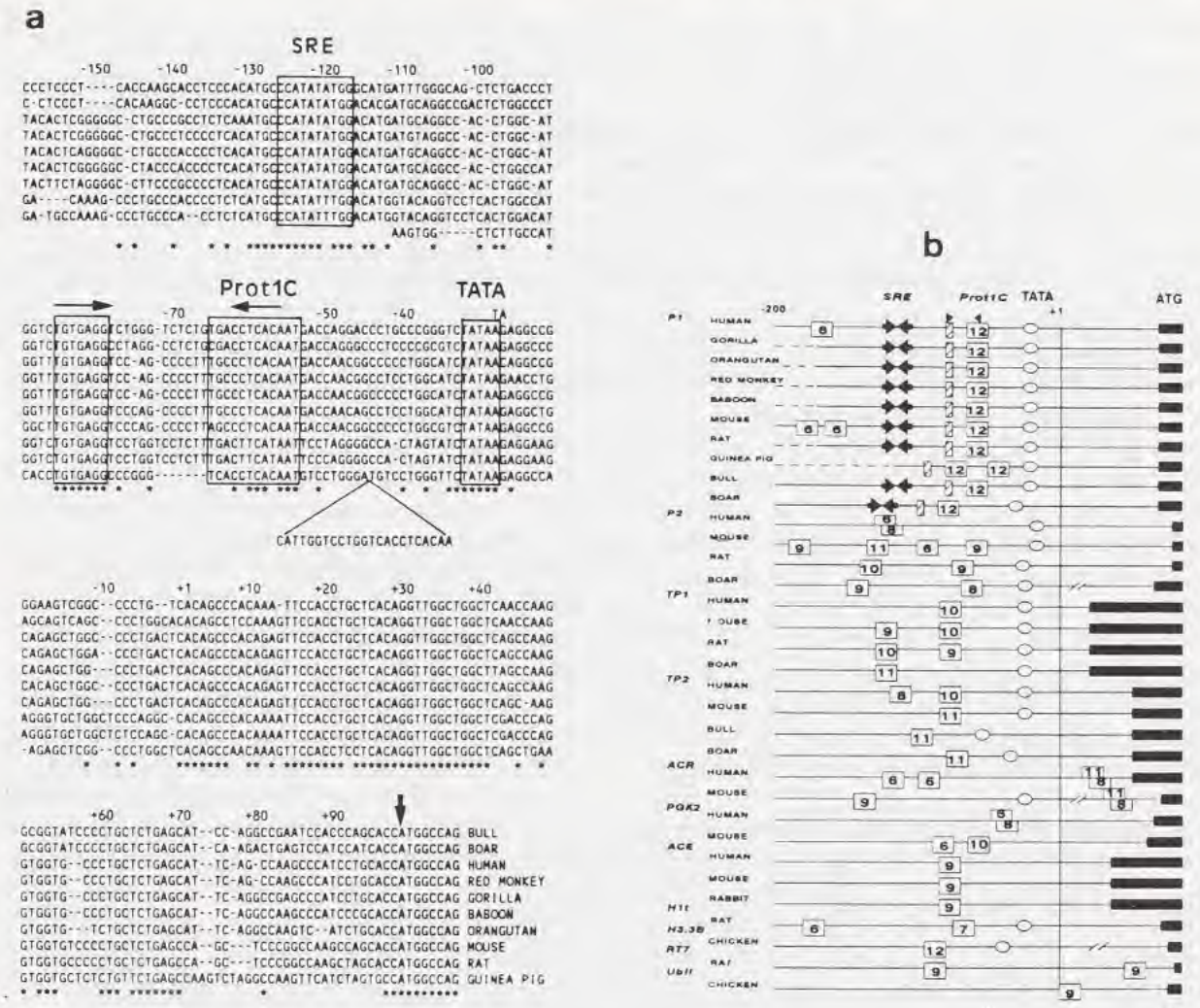


FIG. 3. — **a**: Alignments of all available mammalian *P1* gene promoter sequences. An asterisk indicates that a position is conserved in all species. The nt positions are referenced relative to the tsp [+1]. The conserved *SRE*, "TGTGAGG", *Prot1C* and the TATA box are boxed. The arrow over the *Prot1C* indicates the sequence which is palindromic with the "TGTGAGG" sequence (also arrowed) The start codon (ATG) is indicated by a downward arrow. After [64]. **b:** Position of the *Prot1C*, *SRE* and "TGTGAGG" sequences present in the *P1* genes and position of Prot1C-like sequences present in other testis-expressed genes. The putative *tsp* is indicated [+1]. The numbered open boxes indicate the position of sequences identical or similar to *Prot1C*. The number in the open box indicates the number of matches to the 12mer *Prot1C* [thus, 12 indicates a perfect match]. The beginning of the coding region is shown by the unnumbered solid boxes 3' to the tsp. A broken line indicates that the sequence is not available. The *SRE* is indicated by the two connected arrows facing each other. The "TGTGAGG" sequence is showed by a shaded box and the position and presence of a TATA box is indicated by the ellipse. After [64].

Transcriptional regulation of protamine P1 genes

Transcription of the avian-mammalian P1 type genes occurs in the post-meiotic, haploid stages of spermatogenesis as determined by Northern blot analysis of RNA from testis at different stages of development or from sorted cells [17, 35, 52, 55], and by *in situ* hybridization [35, 38, 44, 55]. Run-off assays on isolated mouse nuclei indicate that the mouse P1 gene is activated at the round spermatid stage [39]. What are the mechanisms leading to this specific activation? The availability of the nucleotide sequences from mouse, human and bull led to the prediction of some potential regulatory elements. For instance a TATA box is present in all of them [18, 24, 32], a

Source : MNHN, Paris

CRE-like element [50], CAT box, CG boxes and additional sequences [18, 24, 32, 50, 52]. A different approach in the prediction of potentially important sequences has been the comparison of homologous or heterologous protamine genes in the search for the conserved regions with the assumption that important regulatory sequences would have been conserved in evolution [50]. Thus the following comparisons were reported: mouse P1 and P2 genes [24]; human, bull and mouse P1 genes [33]; human P1 and P2 genes, mouse P1, bull P1 and human P1 [18]; porcine genes [26]; and chicken, bull P1, mouse P1 and trout protamine [50]. A common problem in all the studies comparing protamine gene P1 sequences was that the homology between the different P1 genes available for analysis was relatively high so that discrimination between conserved regulatory sites and sites conserved simply because of a close origin in evolution was not possible in many cases. This problem was solved when the promoter region of additional P1 genes was sequenced thus increasing the total number of sequences available for comparison [64] (Fig. 3).

Four highly conserved sites were detected in the 5´region (-160 to -1) of the protamine genes [64]. The first one (-29 to -35) corresponds to the already previously described TATA box, but with the novelty of being preceded invariably in all species by the di-nucleotide TC (Fig. 3). The second conserved region (-55 to -66) was named Protamine 1 Consensus (Prot1C) which is a CRE-like sequence (but always differently). The relevance of this conserved element in the expression of the P1 genes is strongly supported by the demonstration of a mouse testis trans-acting factor ([75], Tet-1) which binds and matches in the mouse the first 11 bp of the corresponding Prot1C sequence. Independent experiments using different oligonucleotides corresponding to the mouse P1 5´ region [82] showed that the region -35 to -70 led to the appearance of three different specific bands in gel retardation assays upon incubation with nuclear extracts from different tissues. Only one of those bands was testis-specific. Similar results have been obtained with the rat P1 sequence using rat nuclear extracts [65]. The third highly conserved region detected in the 5´of all P1 genes corresponds to the sequence TGTGAGG (-88 to -82). This sequence is a palindrome of the seven central nt of Prot1C (binding sequence for factor Tet1 in the mouse) and is exclusively present in this region in all P1 genes whose sequence is available suggesting an important function in the differential expression of the protamine P1 genes. This region corresponds to box E in the mouse promoter [24]. The fourth highly conserved region corresponds to MATGCCCATATWTGGRCAYG and has the typical structure of serum response elements (SRE) [64]. This region demonstrates specific interaction with a factor present in rat nuclear extracts from different tissues with a distinctive shifted band appearing in the testis extracts [65]. Also the equivalent region in the mouse P1 gene (described as Box O) demonstrates specific binding with a factor present in mouse nuclear extracts. Thus two highly conserved sequences, the Prot1C (also referred as to CRE-like in all P1 sequences or Tet1 binding site in the mouse), and the SRE appear to bind a testis specific factor or a testis-specific combination of factors [65]. Both of these sequences lie within the minimal region (from bp -150 to bp -37) described to direct spermatid-specific expression with a heterologous promoter from a human growth hormone reporter gene [56, 81, 82].

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Source : MNHN, Paris

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Source : MNHN, Paris

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Source : MNHN, Paris

A

Acanthobothrium 59
Acanthobothrium filicolle benedenii 89
Acanthobothrium filicolle filicolle 89
Acanthochitona pygmaea 132
Acanthochitona viridis 132
Acanthochitonida 146
Acanthochitonidae 132
Acanthocotyle 58
Acanthodasys 108
Acanthopleura granulata 132, 134
Acanthopleuridae 146
Acanthurus monroviae 57
Acarus siro 223
Acipenser 346
Acipenser sturio 346
Acmaeidae 171
Acoela 38, 39
Acomys 409, 410, 417, 418
Acotylea 41
Acrosterigma reeveanum 157
Actinodactylella 47
Actinodactylella blanchardi 46
Actinodactylellidae 41, 46
Aculifera 145
Adelotus brevis 322
Adlerocystis 224
Aegla 252
Agama adramitana 367
Agama agama 367
Agama stellio 367
Agamidae 367
Agelaius phoeniceus 438, 440, 441
Agnathans 506
Alaria 58
Alces alces 539
Algyroides alleni 364
Aliaporcellana 258
Aliaporcellana suluensis 254
Allogalathea 258
Allogalathea elegans 253
Alopias pelagicus 314, 316, 317
Alopiidae 316
Alouatta seniculus 539, 540
Alytes 328, 330, 346, 352
Alytes obstetricians 322
Amblychelipas javanicens 169
Amblychilepas 172
Ambystoma maculatum 336
Ambystomidae 336
Amniotes 343, 344, 355
Amphibdella 58
Amphibia 321, 333
Amphilina 59, 73
Amphilinidea 69, 73
Amphisbaena darwinii 360
Amphiscolops 39
Amphiuma tridactylum 336
Amphiumidae 336
Anas platyrhynchos 346
Ancylostoma caninum 122
Angiostrongylus cantonensis 122
Anguimorpha 380
Annulipalpia 306
Anolidae 367
Anolis carolinensis 367
Anomalopus verreauxii 360
Anomura 253, 254, 256
Anopheles maculipennis 306
Anoplodiscus 58
Anoplodiscus cirrusspiralis 48
Anoplura 304
Anthocidaris crassispina 16, 17
Anthopharynx sacculipenis 43
Anthopleura 18
Anthopleura midori 16, 17
Anura 328, 336, 337
Apatemon 58
Apatemon graciliformis 55, 57, 61
Aphalloides 58, 63, 69
Aphalloides coelomicola 55, 57, 61
Aphelasterias japonica 477, 484
Aphelenchoides blastophthorus 122
Apis mellifera 464
Aplacophora 137
Aporina 59
Aporina delafondi 88, 89, 90
Aporocotyle 58, 63
Aporocotyle spinosicanalis 55, 57, 62
Aprasia repens 372
Arbacia lixula 484
Arbacia punctulata 477, 484
Archaeobrachyura 276
Archaeogastropoda 180
Archeognatha 304
Arcticoidea 160
Aristaeomorpha foliacea 232, 234, 236, 237, 239, 243, 244, 246, 248, 249
Aristeidae 232, 234
Aristeus antennatus 232, 234, 236, 237, 239, 243, 244, 248, 249
Artemesia 233
Arthroleptella 337
Arthroleptella lightfooti 334, 336, 337
Ascaphus 328, 334, 340, 346, 352, 377
Ascaphus truei 322, 464
Ascaris megalocephala 122,124
Ascaris suum 122
Ascidia 17, 466
Ascidia callosa 503
Ascidia sydneiensis semea 16, 17
Asphondylia ruebsaameni 306
Aspiculuris tetraptera 122

Source : MNHN, Paris

Aspidites 374, 376
Aspidites melanocephalus 372, 374
Aspidogaster 58, 71
Aspidogastrea 71, 73
Asterias rubens 480
Asterias vulgaris 477, 480
Atriaster (Atriaster) 58
Atriaster (Atrispinum) 58
Austramphilina 59
Austrocochlea obtusa 169
Aves 355
Avitellina 59
Avitellina centripunctata 89
Axiidae 254
Axine 58
Axioidea 253, 254
Axius 258
Axius glyptocercus 252, 254

B

Babinka 162
Bactrocera (=Dacus) oleae 306
Balanus 18
Balanus nubilus 464
Balanus uliginosus 16, 17
Baltoplana magna 44
Barbastella 421, 424
Barbastella leucomelas 422, 425
Bathydrilus formosus 192
Batillus cornutus 169
Batoids 319
Bdella septentrionalis 223
Bibio 304, 306
Bibionidae 306
Bilateria 148
Birgus 256
Birgus latro 253, 256
Bivalvia 180
Blattodea 304
Boidae 376
Boiga irregularis 372, 374
Boletina 303, 304, 306, 308
Boltenia villosa 503
Bombina 327, 328, 330, 346, 352
Bombina orientalis 322, 324, 327, 328
Bombina variegata 322
Bombyliidae 306
Bombylius 306
Bos taurus 453, 539
Bothrimonus 59
Bothrimonus sturionis 89
Bothriocephalus 59
Bothriocephalus clavibothrium 89
Bothromesostoma personatum 43
Bovine 516
Brachycera 306
Brachylaimus 58
Brachyura 265, 270
Bradynectes sterreri 39
Bradypodion karroicum 359, 360, 363, 364, 368, 370, 376
Branchiostoma 505
Branchiostoma floridae 451, 467, 504, 505
Bresslauilla relicta 46, 48
Breviceps 337
Breviceps gibbosus 334, 336, 337, 338
Brugia pahangi 122
Bucephaloides 58
Bufo 327, 334, 339, 455, 463, 465, 466
Bufo arenarum 322
Bufo bufo gargarizans 322, 324, 327, 328
Bufo fowleri 455
Bufo japonicus 453, 464, 465
Bufo marinus 322
Bufo rangeri 336, 337, 338
Bufonidae 336
Buthus 216
Buthus occitanus 206
Byrsophlebidae 41

C

Caballerocotyla 58
Caecilians 337
Caeciliidae 336
Caenorhabditis elegans 25, 30, 124
Caiman crocodylus 346, 347, 348, 349, 355
Cairina moschata, 57
Calceostoma 58, 73, 90
Calcinus 256, 260
Calcinus gaimardii 254
Calcinus laevimanus 254
Calcinus minutus 254, 258
Calicotyle 47, 58, 73
Callianassa 258
Callianassa arenosa 254
Callianassa australiensis 252, 254
Callianassidae 254
Callianassoidea 253, 254
Calliotropis glyptus 169
Calliphora vomitoria 306
Calliphoridae 306
Callista chione 158, 455
Callistoplacidae 132, 146
Callistoplax crassicostatus 132
Callochiton castaneus 132, 136
Callochitonidae 132, 146
Callophrys 18
Callophrys ferrea 16
Calocarcinus 280
Calocarcinus africanus 268, 275
Calocarididae 253
Calocaris macandreae 253
Caluromys philander 348
Camelus dromedarius 539
Cancellus 254, 256, 258, 260
Capillaria hepatica 122
Capra hircus 539
Carabus catenulatus 286

Source : MNHN, Paris

Carassius auratus 450, 464
Carcharhinidae 316
Carcharhiniformes 316, 319
Carcharhinus plumbeus 316, 317
Carcharias taurus 314, 316, 317
Carcinoscorpius 204, 210, 216
Carcinoscorpius rotundicauda 216
Cardinalis cardinalis 438, 440, 441
Cardioidea 160
Cardita muricata 158
Carditidae 162
Carditoidea 162
Cardium edule 158
Carlia 361, 362, 375, 379, 380
Carlia pectoralis 361, 362, 372, 374, 376
Cataetyx laticeps 464, 465, 467, 509, 510
Catagapetus nigrans 306
Catenotaenia 59
Catenotaenia pusilla 89
Catenulida 38
Caudata 466
Cavia porcellus 539
Cecidomyiidae 306
Cellana capensis 170
Cellana testudinaria 170
Cellana toreuma 170
Cemocotyle 58
Centrophorus squamosus 507
Centrophorus uyato 507
Centroscymnus coelolepis 507
Centroscymnus crepilater 507
Centroscymnus owstoni 316, 317, 319
Centurus carolinus 440
Cephalobus quinilineatus 122
Cephalochordates 505
Cephalodasys 116
Cephalodasys maximus 106, 112, 116
Cephaloscyllium umbratile 316, 317, 318
Cerastoderma edule 180, 182
Ceratitis capitata 285, 306
Cercomeridea 56, 69
Cercopithecus patas 539, 540
Cerris puludum 286
Cervus elaphus 539
Chaetoderma 145
Chaetoderma argenteum 132, 138, 140, 144
Chaetoderma canadense 132, 138, 140
Chaetodermomorpha 132, 140, 145, 146
Chaetonotida 106, 112, 116
Chaetonotidae 106
Chaetonotus maximus 106, 108
Chaetopleura apiculata 132, 137
Chaetopleuridae 132, 146
Chaetopteryx gessneri 306
Chalcides 359, 379
Chalcides ocellatus 360, 370, 376
Chalcides ocellatus tiligugu 360, 362, 362
Chama macerophylla 158
Chamaeleonidae 368
Chamoidea 160
Chara corallina 469
Chartoscirta cincta 286
Cheiridium museorum 207
Chelicerata 203
Chelonia 355, 376
Chelyosoma productum 454, 458, 503
Cherax destructor 47
Childia groenlandica 39
Chimaera phantasma 315, 316, 317
Chimaeridae 316
Chimaeriformes 316
Chiromantis 337, 340
Chiromantis xerampelina 333, 336, 337, 338, 341
Chironomidae 306
Chironomus 306
Chiroptera 421
Chirostylidae 254, 258
Chiton tuberculatus 132, 137
Chitonidae 132, 146
Chlamydia trachomatis 463, 464, 470
Chlamydomonas 15, 16, 17, 18, 20, 21, 28
Chlamydomonas reinhardti 16, 17
Chlamydoselachidae 316
Chlamydoselachus anguineus 313, 314, 316, 317
Chlorostoma argyrostoma lischkei 169
Chondrichthyes 507
Choricotyle 58
Chrysomya megacephala 285
Chthonerpton indistinctum 336
Cichlidogyrus 58
Circe cf *plicatina* 157, 160
Cleistogamia longicirrus 46
Cleithrarticus 55, 57, 58, 67, 68
Clibanarius 256
Clibanarius corallinus 253
Clibanarius erythropus 253
Clibanarius longitarsus 253
Clibanarius misanthropus 253
Clibanarius nathi 253
Clibanarius taeniatus 253, 268, 270, 271
Clibanarius virescens 253
Clitellio 194, 196, 198
Clitellio arenarius 192, 196
Clonorchis 58
Cnemidocarpa finmarkiensis 503
Cnemidophorus 364, 370, 375, 379, 380
Cnemidophorus lemniscatus lemniscatus 364
Cnemidophorus sexlineatus 347, 362, 366, 368, 370, 374, 376
Codakia punctata 156, 157, 160
Coelogynoporidae 41
Coenobita 256
Coenobita brevimanus 254
Coenobita clypeatus 253
Coenobita perlatus 254, 258
Coenobita purpureus 254
Coenobita rugosus 253
Coenobita spinosus 253
Coenobita variabilis 253
Coenobitidae 253, 254, 256
Coleoptera 297, 304
Collyricloides 58

Source : MNHN, Paris

Coluber constrictor 372
Coluber viridiflavius viridiflavius 372
Colubridae 376
Concinnocotyla 58
Constrictor 372
Constrictor constrictor 372
Coralliodrilus 190, 196, 197, 198
Coralliodrilus rugosus 189, 190, 191, 192, 195
Corbicula 158, 162, 163
Corbicula fluminea 158, 162
Corbicula sandai 158
Corbiculoidea 160
Coronella austriaca 372
Corrigia 58
Cosmopolites sordidus 297, 298, 299
Cotugnia 59
Cotugnia polyacantha 89
Coturnix coturnix 346
Coturnix japonica 539, 540
Cotylea 41
Cotylophorum 58
Craspedella 46
Crassatellidae 162
Crassostrea 17
Crassostrea angulata 180, 182, 184
Crassostrea gigas 16, 17
Crenilabrus cinereus 57
Crenomytilus grayanus 504
Cricetomyinae 414
Cricetulus migratorius 539
Crocodilia 355
Crocodylus 349
Crocodylus johnstoni 344, 346, 347, 348, 349, 350, 352, 355
Crotalus adamanteus 372
Crustacea 231, 243, 251, 265
Crypthecodinium cohnii 463, 464, 470
Cryptoblepharus virgatus 361, 362, 372, 374
Cryptobranchidae 336
Cryptobranchus 341
Cryptobranchus alleganiensis 336
Cryptocellus 216
Cryptocellus boneti 207, 208, 211, 214
Cryptochiton stelleri 129, 130, 132, 142
Cryptocotyle 58
Ctenotus 362, 379, 380
Ctenotus robustus 360, 362, 376
Ctenotus taeniolatus 360, 362
Culex pipiens 304, 306
Culicidae 306
Cupriguanus 367
Cupriguanus scapulatus 366
Curculio elephas 299
Cyclodorippoidea 276, 277
Cylindrotaenia 59
Cylindrotaenia hickmani 89
Cymonomus 268, 273, 277
Cynopterus brachyotis 422
Cyrnus trimaculatus 306
Cyta latirostris 218

D

Dagnaudus (=Paramola) petterdi 268, 276
Dalatias licha 313, 315, 316, 317
Dalyelliida 41, 44, 74
Dalyelliidae 41, 44
Dardanus 254, 256
Dardanus arrosor 253, 258
Dardanus crassimanus 253
Dardanus lagopodes 254
Dardanus megistos 253
Dardanus scutellatus 254
Deania 318
Deania calcea 316, 317
Deania historicosa 316, 317
Deania profundorum 507
Decadidymus gulosus 46, 49
Decapoda 231, 243, 251, 265
Delma tincta 372
Dendrobranchiata 231, 243
Dendrobranchiate 233, 236
Dendrofissurella 172
Dendrofissurella scutellum 169
Dendromurinae 414
Dendromus 409
Dendromus mesomelas 410, 414
Dendromus mystacalis 410, 414, 416
Deomys 409, 417, 418
Deomys ferrugineus 410, 414, 416
Deontostoma californicum 122
Dermaptera 304
Dermestes frischii 299
Dicentrarchus labrax 509, 510
Diceratocephala boschmai 46
Diclidophora 58, 73
Dicrocoelium 58
Dictyostelium 23, 24, 28, 32, 34
Dictyostelium discoideum 23, 24, 26, 27, 28, 29,30, 31, 32, 33, 34
Dictyostelium giganteum 26, 31
Dictyostelium mucoroides 26
Dictyostelium purpureum 26, 31
Dicyema misakiensis 21
Didelphis marsupialis 539
Didymorchidae 41, 47
Didymorchis 47
Didymozoon 58, 69, 70
Diemictylus 341
Digenea 59, 69, 70, 73
Dioctophyma renale 122
Diodora aspera 169
Diogenes 256, 260
Diogenes custos 253
Diogenes miles 253
Diogenes pallescens 254
Diogenidae 253, 254, 256
Dionchus 58
Dipetalonema dessetae 122
Dipetalonema setariosum 122
Dipetalonema viteae 122, 124
Diphyllobothrium 59

Source : MNHN, Paris

Diphyllobothrium latum 89
Diplectanum 58, 73
Diplodasys ankeli 106, 108, 110, 112
Diplozoon 58, 63, 64, 71, 72, 74
Diplozoon gracile 55, 57, 64
Diplozoon nipponicum 64
Diplura 304
Diptera 301, 302, 304, 308
Dirofilaria immitis 122
Discoglossus 327, 328, 330, 346, 352
Discoglossus pictus 322
Divales bipustulatus 299
Divariscintilla 155, 156, 160, 163
Divariscintilla troglodytes 158
Divariscintilla yoyo 158
Dixa 306
Dixidae 306
Donacidae 160
Donax 163
Donax deltoides 157, 160, 163
Donax madagascariensis 158, 163
Donax serra 158, 163
Donax sordidus 158, 163
Donax trunculus 158, 163, 180, 182, 455
Doliopharyngiophora 74
Draculiciteria 108
Dreissena polymorpha 158
Dreissenoidea 160
Dromaius novaehollandiae 346
Dromiacea 272
Dromidia antillensis 281
Dromidiopsis 272, 274, 276, 277
Dromidiopsis edwardsi 268, 270, 271, 272
Dromiidae 272
Drosophila 102
Drosophila melanogaster 285, 306
Drosophilidae 306
Drymarchon corais 372
Duthiersia 59
Duthiersia fimbriata 89
Dynomene 272, 274
Dynomene aff. *devaneyi* 265, 266, 267, 268, 270, 272, 276, 278
Dynomenidae 274
Dysdera 210

E

Echeneibothrium 55, 57, 59, 68
Echeneibothrium beauchampi 89
Echinobothrium 59
Echinobothrium affine 89
Echinobothrium brachysoma 89
Echinobothrium harfordi 89
Echinobothrium typus 89
Echinococcus 59
Echinococcus granulosus 89
Echinoderms 475, 476, 491
Echinoida 466
Echinostoma 58, 97, 102
Echinostoma caproni 98, 100
Echinostoma liei 98, 101
Echinostoma togoensis 55, 57, 61
Echymipera kalubae 398
Echymipera rufescens 398
Ekphymatodera thomasoni 122
Elapidae 376
Eledone 451
Elenchus japonicus 291, 292
Elenchus tenuicornis 291, 292
Elephas 539
Emballonura furax 427
Emberizidae 440
Embioptera 304
Emerita 260
Emerita analoga 253
Emerita asiatica 253
Emerita talpoida 253
Empididae 306
Emydura 349
Emydura kreffti 344
Encotyllabe 58
Enoplus anisospiculus 122
Enoplus demani 122
Ensis ensis 158, 455
Ensis minor 451
Entobdella 58
Eonycteris spelaea 422
Ephemeroptera 304
Epicrius mollis 218
Epigonichtys 505
Epimenia 138, 140
Epimenia australis 132, 138, 143
Equus caballus 539
Erpocotyle 58
Escherichia coli 470, 493
Etisus 280
Etmopterus 313, 316, 318
Etmopterus brachyurus 316, 317
Etmopterus molleri 316, 317
Etmopterus pusillus 316, 317, 507
Eucarida 236
Eucestoda 69, 72, 73
Eucidaris 476
Eucidaris crassa 480
Eucidaris esculentus 479, 480
Eucidaris tribuloides 476, 477, 484
Eucrassatella cumingii 158
Eucrassatella kingicola 158
Eudromia elegans 346
Euglena gracilis 470
Eukalyptorhynchia 41, 43
Eumeces laticeps 360, 362
Eumunida 258
Eumunida sternomaculata 254
Eupagurus angulatus 253
Eupagurus bernhardus 253
Eupagurus prideauxii 253
Euphausia 236
Eurytrema 58
Eusimonia mirabilis 214

Source : MNHN, Paris

Eutheria 466
Euzetrema 58
Exechia seriata 303, 304, 306

F

Fasciola 58
Fecampiida 41, 47
Fecampiidae 41
Felis catus 539
Fibricola 58
Filistata 211
Filistata insidiatrix 208, 211
Fissurella 172
Fissurella mutabilis 169
Fissurella natalensis 169
Fissurellidae 169, 172
Fissurelloidea 169
Forcipulata 466
Fragum hemicardium 157
Fragum unedo 157, 160
Fulvia tenuicostata 158
Furnestinia 58, 68
Furnestinia echeneis 55, 57, 66

G

Galathea squamifera 253
Galathea strigosa 253
Galatheidae 253, 258
Galatheoidea 253, 254
Galeocerdo cuvier 316, 317
Galeommatoidea 160
Galeus 318
Galeus eastmani 316, 317
Galeus melastomus 507
Galeus nipponensis 315, 316, 317
Gallus domesticus 346, 442, 453, 538, 539, 540, 541
Ganeo 58
Gasterophilidae 306
Gasterophilus intestinalis 306
Gasterosteus 465, 466
Gastrocotyle 58, 73
Gastromermis 122, 124, 125
Gastropoda 180
Gastrotricha 105
Gazella dorca 539
Gebia littoralis 253
Gekkonidae 370
Gekkota 380
Geocentrophora 42
Geocentrophora intersticialis 42
Geocentrophora wagini 42
Geocentrophora wasiliewi 42
Geopelia striata 344, 346, 347, 348, 349, 350
Gerbillinae 412
Gerbillurus 409
Gerbillurus paeba 410, 412, 413, 417
Gibbula cineraria 180, 182
Gibbula divaricata 453
Gibbula tumida 169
Gibbula umbilicalis 169, 180, 182
Gieysztoria 44, 46
Glaridacris 59
Glaridacris catostomi 89, 90
Glauconome 157, 160
Glossosomatidae 306
Glyphotaelius pellucidus 306
Gnathostoma 122
Gobio gobio 57
Gobius micropus 57
Goeridae 306
Gonapodasmius 58, 69
Gonoplasius 58, 73
Gorgodera 58, 97, 98, 99
Gorilla gorilla 539
Gotocotyla 58, 63, 71
Gotocotyla acanthura 65
Graffilla buccinicola 46
Graffillidae 41, 46
Grus vipio 346
Gymnophiona 336
Gynaecotyle 58
Gyratrix 43
Gyrocotyle 59, 73
Gyrocotylidea 69, 73
Gyrodactylus 58

H

Haematoloechus 55, 57, 58, 61, 63, 69, 97, 98, 99
Haemonchus contortus 124
Halichondria 18
Halichondria japonica 16, 17
Halictophagus chilensis 291, 292
Haliotidae 169, 172
Haliotis discus 169
Haliotis diversicolor aquatilis 169
Haliotis midae 169
Haliotis rufescens 169
Haliotoidea 169
Hanleyella asiatica 137
Haplobothrium 59
Haplobothrium globuliforme 89, 90
Helcion dunkeri 170
Helcion pectunculus 170
Helcion pellucidus 170, 180, 182
Helcion pruinosus 170
Heleophryne 340, 341
Heligmosomoides polygyrus 119, 120, 122, 123, 124
Heligmosomoides polygyrus bakeri 120
Hemicentrotus 17
Hemicentrotus pulcherrimus 16, 17, 18
Hemidactylus frenatus 370
Hemidactylus mabouia 370
Hemiptera 285, 304
Hemisotidae 336
Hemisus marmoratus 336, 337
Hemitriakis japanica 316, 317, 319

Source : MNHN, Paris

Heptathela 210, 212, 213
Heptathela kimurai 210, 211, 212, 213, 218
Heterakis gallinarum 122, 124
Heteraxine 58
Heteraxinoides 55, 57, 58, 63, 64
Heterocotyle 58
Heterodera (Globodera) rostochiensis 122
Heterodera (Globodera) virginiae 122
Heterodera (Heterodera) avenae 122
Heterodera (Heterodera) schachtii 122
Heterodonta 180
Heterodontidae 316
Heterodontiformes 316, 319
Heterodontus japonicus 314, 316, 317
Heterodrilus 189, 190, 198
Heterodrilus minisetosus 190, 191, 192
Heterodrilus pentcheffi 190, 191, 192, 195
Heteronotia 370, 380
Heteronotia binoei 346, 347, 368, 369, 370, 374, 376
Heterorhabditis bacteriophora 122
Heterotremata 277
Heteroxenotrichula 108
Heteroxenotrichula squamosa 106, 108, 112, 115
Hexanchiformes 316, 319
Hexostoma 58
Hippa 260
Hippa pacifica 254, 258
Hippidae 253, 254, 258
Hippoidea 253, 254
Hirundinidae 439
Holothuria 466
Holothuria tubulosa 477, 484
Holothuria tubulosa 491, 492, 493, 494, 495, 496, 498
Homo sapiens 539
Homola ranunculus 268, 276
Homolidae 276
Homolodromia 265, 266, 272, 274, 277, 281
Homolodromia kai 265, 266, 267, 268, 270, 272, 274, 276, 278
Homolodromiidae 272
Homologenus 276
Homolomannia sibogae 276
Homoptera 304
Hydrolagus colliei 503, 507, 508
Hydrolagus mitsukurii 316, 317
Hydropsyche pellucidula 306
Hydropsychidae 306
Hydroptila aegyptia 306
Hydroptila angulata 306
Hydroptila forcipata 306
Hydroptila tineoides 306
Hydroptilidae 306
Hyla 321, 327, 328
Hyla japonica 322, 324, 327, 328
Hyla meridionalis 322
Hyla rubra 334
Hylobates lar 539
Hymenochirus 467
Hymenolepis 59
Hymenolepis diminuta 89
Hymenolepis microstoma 89
Hymenolepis nana 88, 89, 90
Hymenoptera 304
Hyperoliidae 336
Hyperolius horstocki 336
Hypoconcha 272, 281
Hypoconcha arcuata 281

I

Ichthyopiidae 336
Integripalpia 306
Ichthyopis glutinosus 336
Icteridae 440
Iguania 379
Iguanidae 366, 376
Iliacantha subglobosa 277
Inanidrilus 198
Inanidrilus bulbosus 191, 192
Inanidrilus leukodermatus 191, 192
Inermicapsifer 59
Inermicapsifer guineensis 89
Inermicapsifer madagascariensis 89
Insecta 285, 291, 297, 301
Isancistrum 58
Ischnochiton albus 132
Ischnochitonidae 132, 146
Isoodon macrourus 398, 405
Isoodon obesulus 398
Isoptera 304
Isurus oxyrinchus 314, 315, 316, 317, 319

J

Jacana jacana 346

K

Kalyptorhynchia 41, 43
Katharina tunicata 132
Keroplatus reaumuri 303, 306
Kronborgia 40, 49, 75
Kronborgia isopodicola 47, 48
Kuhnia 58

L

Lacerta laevis 364
Lacerta lepida 364
Lacerta lepida lepida 364
Lacerta sicula campestris 364
Lacerta taurica 364
Lacerta viridis 364
Lacerta vivipara 347, 364
Lacertidae 364, 376
Lacistorhynchus 59
Lacistorhynchus tenuis 89, 90
Lagarocotyle 58

Source : MNHN, Paris

Lamellodiscus 58
Lamnidae 316
Lamniformes 316, 319
Lampropeltis getulus 372, 374
Lampropholis 362, 379, 380
Lampropholis delicata 344, 349, 361, 362, 367, 372, 374, 376
Lasaea 155, 156, 163
Lasaea subviridis 158
Latimeria 352, 354
Latimeria chalumnae 346
Latreillia 265, 266, 268, 271, 276, 277, 281
Latreilliidae 277
Latreillopsis gracilipes 268, 271, 276
Lecithoepitheliata 41, 42
Lepetodrilidae 169, 172
Lepetodriloidea 169
Lepetodrilus fucensis 168, 169, 172
Lepidochitona 131
Lepidochitona dentiens 132, 134, 137
Lepidochitona fernaldi 132, 137
Lepidochitonidae 146
Lepidodasyidae 106
Lepidodermella squamata 106, 108
Lepidopleuridae 132, 146
Lepidoptera 304
Lepidozona retiporosa 132, 134
Leptoceridae 306
Leptocerus tineiformis 306
Leptochiton 140
Leptochiton asellus 129, 130, 132, 136, 137, 141, 142
Leptocoris trivittatus 286
Leptodrusus budtzi 306
Leptopelis flavomaculatus 336, 337
Lialis 359, 375, 380
Lialis burtonis 347, 367, 370, 371, 372, 376, 380
Lilium longiflorum 469, 470
Limnephilidae 306
Limnephilus bipunctatus 306
Limnephilus rhombicus 306
Limnodriloides gurwitschi 199
Limnodriloidinae 191, 192
Limnodynastes 327
Limnodynastes peronii 322
Limulus polyphemus 205, 213, 214, 216, 223, 450
Liolaemus 367
Liolaemus austromendocinus 366
Liolaemus weigmanii 360
Liophis miliaris 372, 374
Liotella parvirota 169
Lithodes 260
Lithodes maja 253
Lithodidae 253
Litoria 327, 465, 466
Litoria caerulea 322
Litoria fallax 322
Litoria gracilenta 322
Litoria lesueuri 322
Litoria peronii 322
Litoria rubella 322
Litosomoides carinii 122
Littorina littorea 180, 181, 182
Littorina saxatilis 180, 181, 182
Littorinidae 466
Loa loa 122
Lobatostoma 58, 71
Loimosina 58
Loligo 449
Loligo pealeii 453
Lomidae 254, 258
Lomis 252, 260
Lomis hirta 254, 258
Lomoidea 254
Lophuromys 410, 417
Lottia digitalis 170
Lottia pelta 170
Lottia strigatella 170
Lottiidae 170, 466
Lucinoidea 160
Lunella cinerea 169
Lunella granulata 169
Luriculus australiensis 46
Luridae 41, 46
Lygodactylus 380
Lygodactylus picturatus 370, 376
Lyreidus 277
Lyreidus brevifrons 268, 273, 276
Lytechinus pictus 470, 477, 480, 484
Lytechinus variegatus 477

M

Macaca mulatta 539
Macoma nasuta 451
Macrodasyida 106, 110, 112
Macrodasyidae 106
Macrodasys 106, 108, 116
Macroglossinae 422
Macroglossus minimus 422, 425
Macrogyrodactylus 58, 73
Macrophthalmus 280
Macrophthalmus crassipes 268, 278
Macroschisma sinense 169
Macrostomida 39
Macrostomum 39, 49
Macrostomum tubum 39
Macrotis 398
Macrotis lagotis 397, 398, 399, 400, 401, 402, 404, 405, 406
Mactroidea 160
Maehrenthalia 43
Majids 268
Malacothrix 409
Malacothrix typica 410, 414, 416, 417
Mallophaga 304
Mammalia 356, 397, 409, 421, 515, 525, 537
Mantodea 304
Marchantia polymorpha 469
Maricola 41
Maritrema 58
Marsupalia 466

Source : MNHN, Paris

Masticophis flagellum flagellum 372
Mastigoproctus giganteus 207, 213
Mathevotaenia 59
Mathevotaenia herpestis 88, 89
Meara 38
Mecoptera 304
Mecynostomum 39
Mecynostomum auritum 39
Megachiroptera 422
Megalocotyle 58, 73
Megaloptera 304
Meiogymnophallus 58
Melampophylax melampus 306
Melanerpes carolinus 346
Meleagris gallopavo 346
Meloidodera floridensis 122
Meloidogyne acronea 122
Meloidogyne arenaria 122
Meloidogyne graminicola 122
Meloidogyne hapla 122
Meloidogyne incognita 122
Meloidogyne oryzae 122
Menaethius monoceros 275
Meretrix 20
Meretrix lusoria 16, 17
Merluccius 511
Merluccius capensis 509, 510
Merluccius merluccius 57
Mesacanthion hirsutum 122
Mesodasys laticaudatus 106, 111, 112
Metagonimus 58
Metamicrocotyla 58
Metapeneopsis 233
Metastrongylus apri 122
Metazoa 17, 18
Metorchis 58
Metridium senile 454, 458, 467
Microcarina surgerea 169
Microchiroptera 422
Microcotyle 58
Microdalyellia 44
Microphallus 58
Micropterna sequax 306
Microstomum 39
Micrurus fulvius 372
Mictyris 280
Mictyris longicarpus 268, 278
Miniopterinae 422
Miniopterus 428
Miniopterus schreibersii 422, 425, 426, 427, 428
Mixophyes fasciolatus 322
Mollusca 129, 155, 167, 179, 451
Molothrus ater 438, 440
Moniezia 55, 59, 68
Moniezia benedeni 89, 90
Moniezia expansa 57, 89, 90
Monobothrium 59
Monobothrium wageneri 89
Monocelididae 41
Monocotyle 58
Monodonta australis 169
Monodonta labio labio 169
Monodonta turbinata 169
Monoecocestus 59
Monoecocestus americanus 89
Monogenea 63, 71, 72, 73
Monopisthocotylea 68, 72, 73
Monopylephorus 198, 199
Monopylephorus limosus 192
Montfortula 172
Montfortula conoidea 169, 172
Mopalia 466
Mopalia ciliata 132, 134, 136
Mopalia lignosa 132
Mopalia muscosa 132, 134
Mopaliidae 132, 146
Mullus 511
Mullus barbatus 454
Mullus surmuletus 458, 509, 510
Multicotyle 58, 71, 73
Multipeniata 42
Munida 254, 258
Munida rugosa 253
Munidopsis 254, 258
Murina leucogaster 422, 425
Murininae 422
Mus musculus 16, 17, 539
Mustelus 318
Mustelus canis 316, 317
Mustelus griseus 315, 316, 317
Mustelus manazo 316, 317
Mycetophilidae 306
Mycrohylidae 336
Myiarchus crinitus 440
Myliobatididae 316
Myliobatiformes 316
Myliobatis tobijei 316, 317
Mylonchulus nainitalensis 122
Myocidaris 476
Myotini 422
Myotis 421, 422, 424, 425, 427
Myotis adversus 422
Myotis formosus 422
Myotis hosonoi 422, 425
Myotis lucifugus 422, 427
Myotis macrodactylus 421, 422, 424, 425, 427
Myotis nattereri 421, 422, 424, 425, 426, 427
Mysella 155, 156, 163
Mysella tumida 158
Mystacides azurea 306
Mystromyinae 412
Mystromys 409, 410
Mystromys alba 410, 411, 412, 417
Mytilidae 466
Mytiloidea 180
Mytilus 449, 451, 458, 485
Mytilus californianus 451, 452, 484
Mytilus edulis 16, 17, 180, 182, 184, 455, 464, 465
Myxinidocotyle 58, 73

Source : MNHN, Paris

N

Nacella delesserti 170
Nacellidae 170
Nangura 349
Nangura spinosa 360, 362, 374
Natrix natrix 372
Natrix tesselata tesselata 372
Neanthes 18
Neanthes diversicolor 16, 17
Necturus maculosus 336
Nemastoma 216
Nemastoma lugubre 206
Nematocera 306
Nematoda 119
Nematodirus battus 122
Nematoplanidae 41
Nematospiroides dubius 120
Nematotaenia 59
Nematotaenia chantalae 88, 89
Nemertoderma 38, 50, 129, 130, 149
Nemertodermatida 38
Neoaplectana intermedia 122
Neobatrachus pelobatiodes 322
Neocarus 207
Neoceratodus 343, 346, 354, 355
Neoceratodus forsteri 344, 354
Neochasmus 58
Neodasyidae 106
Neodermata 75
Neodasys 106, 108, 116
Neodorippe 265, 266, 278, 280
Neodorippe astuta 268, 275, 278
Neodorippe callida 275, 278
Neomeniomorpha 132, 138, 140, 146
Neopolystoma 58
Nepa rubra 286
Nerita picea 138
Nerodia sipedon 372, 374
Nicoletiella lutea 205
Nippostrongylus 124
Nippostrongylus brasiliensis 122, 124
Noctiluca 470
Notonecta glauca 286
Notoplana 464
Notoplana japonica 42
Nucella lapillus 180, 181, 182
Nucellidae 466
Numida meleagris 346
Nuttalina flexa 132
Nyctalus 421, 424, 427
Nyctalus noctula 422
Nymphon 204, 205, 208
Nymphon gracile 216
Nymphon rubrum 216

O

Octomacrum 58
Ocyphaps lophotes 349
Ocypode 280
Ocypode ceratophthalma 268, 278
Odocoileus virginianus 539
Odonata 304
Odontaspididae 316
Odontoceridae 306
Odontocerum albicorne 306
Odontophrynus 327
Odontophrynus cultripes 322
Odontorhynchus 43
Oecetis furva 306
Olavius planus 192
Oligochaeta 189
Omphalus pfeifferi 169
Onchobothrium 59
Onchobothrium uncinatum 89
Oncorhyncus keta 453
Onithochiton quercinus 132
Oochoristica 59
Oochoristica agamae 89
Opilioacarus 207
Orcinus orca 539, 540
Orectolobidae 316
Orectolobiformes 316, 319
Orectolobus japonicus 314, 315, 316, 317
Orfelia 302, 303, 306
Ornithodorus tholozani 205
Ornithorincus anatinus 539
Orthoptera 304
Orthotrichia costalis 306
Oryctolagus cuniculus 539
Osteichthyes 508
Ostrea edulis 180, 182, 184
Ostreoidea 180
Otomyinae 416
Otomys 409, 417
Otomys irroratus 410, 416
Otoplanidae 41
Ovis aries 539
Oxystele impervia 169
Oxystele sinensis 169
Oxystele tabularis 169
Oxystele tigrina 169
Oxystele variegata 169
Oxyuranus 376
Oxyuranus microlepidotus 372

P

Pachymedusa 327
Pachymedusa dacnicolor 322
Pagellus acarne 509, 510
Paguridae 253, 254, 256
Paguristes maculatus 253
Paguristes oculatus 253
Paguroidea 253, 254
Pagurus 252, 256, 260
Pagurus bernhardus 253, 268, 270
Pagurus chevreuxi 254, 258
Pagurus excavatus 253

Source : MNHN, Paris

Pagurus hirtimanus 254
Pagurus prideaux 253
Pagurus punctulatus 253
Pagurus striatus 253
Paleognathae 466
Paludicola 41
Pan paniscus 539
Pan troglodites 539
Panagrellus silusiae 122
Pantodon 349, 352
Panulirus 234, 239
Paphia 157
Papio doguera 539
Paradynomene 272, 274, 277
Paradynomene tuberculata 268, 270, 271, 272
Paragonimus 58
Paramacrostomum tricladoides 39
Paramecium 15, 16, 17, 18
Paramecium caudatum 16, 17
Paramola bathyalis 276
Parapaguridae 254, 256
Parapeneus 233
Parapeneus longirostris 232, 233, 234, 236, 239, 249
Parasitus berlesei 218
Paravortex 46, 47
Paravortex cardii 46
Paravortex karlingi 46
Paravortex tapetis 46
Parechinus angulosus 477, 480
Paridae 439
Paromolopsis boasi 276
Parus bicolor 439
Passer domesticus 438, 439, 441, 442
Passeridae 439
Passeriformes 437
Patella aphanes 170
Patella argenvillei 170
Patella aspera 170
Patella barbara 170
Patella caerulea 170
Patella candei 170
Patella canescens 170
Patella cf *miniata* 170
Patella chapmani 170
Patella cochlear 170
Patella compressa 170
Patella concolor 170
Patella depressa 170
Patella ferruginea 170
Patella flexuosa 170
Patella granatina 170
Patella granularis 170
Patella laticostata 170
Patella longicosta 170
Patella miliaris 170
Patella miniata 170
Patella obtecta 170
Patella oculus 170
Patella peronii 170
Patella rustica 170, 180, 182
Patella safiana 170
Patella tabularis 170
Patella vulgata 170
Patellidae 168, 170
Patellogastropoda 168, 174, 180
Patelloida 174
Patelloida profunda albonotata 170, 171
Patelloida saccharina lanx 170, 171
Patiria miniata 477, 484
Pecten maximus 455
Pectinodrilus 190, 197
Pectinodrilus molestus 189, 190, 192, 195
Pelusios adansoni 57
Penaeidae 232, 233
Penaeus 233, 234, 239
Penaeus aztecus 232, 233
Penaeus japonicus 232, 233, 234
Penaeus kerathurus 232, 233, 234
Penaeus setiferus 232, 233
Penaeus vannamei 232, 233
Peneopsis 233
Peneopsis serrata 232, 233, 239
Perameles gunni 398
Perameles nasuta 398
Peromyscus maniculatus 120
Perorcytes longicauda 398
Perotrochus quoyanus 169, 171
Perotrochus westralis 169, 171
Petrolisthes 258, 260
Petrolisthes armatus 254
Petrolisthes lamarckii 253
Petromyzon marinus 450, 467, 505, 506, 508
Phaenocora 48
Phaenocora anomalocoela 43
Phallodrilinae 190, 191, 192
Pharyngostomoides 58
Phasianellidae 169, 172
Phasmida 304
Philopotamidae 306
Philopotamus 308
Philopotamus ludificatus 306
Philopotamus montanus 306
Phlebobranchiata 466
Phodopus sungorus 539
Pholcus 208, 216
Pholcus phalangioides 207, 208, 210, 211, 216
Phyllobothrium 59
Phyllobothrium gracile 89
Phymaturus 366
Phymaturus palluma 366
Physaloptera 122
Phytoptus avellanae 223
Pilodius 280
Pilodius areolatus 268, 275
Pipa 467
Pipidae 336
Pipistrellini 422
Pipistrellus 421, 424, 427
Pipistrellus abramus 421, 422, 424, 425, 426, 427
Pipistrellus abramus abramus 427
Pipistrellus abramus javanicus 427
Pipistrellus avii 422

Source : MNHN, Paris

Pipistrellus endoi 422, 424, 425
Pipistrellus javanicus 422, 424, 425
Pipistrellus javanicus javanicus 427
Pipistrellus pipistrellus 427
Piranga rubra 439
Pisidia 258
Pisidia longicornis 253
Placamen calophyllum 157
Planipennia 304
Planoceros 18
Planoceros reticulata 16, 17
Platyhelminthes 37, 55, 69, 74, 87, 97, 149
Plecoptera 304
Plecotini 422
Plecotus 421, 424
Plecotus auritus 422, 425
Plectanocotyle 58
Plectrocnemia geniculata 306
Plethodon albagula 336, 352
Plethodontidae 336
Pleurodeles 327
Pleurodeles waltlii 336
Pleuroncodes planipes 253
Pleurotomaria africana 171
Pleurotomariidae 169, 171
Pleurotomarioidea 169
Podarcis 364
Podocotyle 58
Podotremata 270
Pogona 359, 360, 368, 375, 379, 380
Pogona barbata 363, 367, 368, 370, 376
Polistes dominilus 292
Polycentropodidae 306
Polycentropus irroratus 306
Polycentropus mortoni 306
Polycladida 41, 42
Polycystis naegelii 43, 44
Polydelphis 122
Polylabroides 58
Polyonyx 258
Polyonyx transversus 254, 258
Polyopisthocotylea 63, 71, 73
Polyplacophora 131, 136
Polysphondylium pallidum 26
Polystoma 58
Polystomoides 58
Pomadasys incisus 57
Pongo pygmaeus 539
Porcellanidae 253, 258
Porcellanopagurus 254, 256, 260
Portunus pelagicus 268, 275, 280
Postharmostomum 58
Potamonautes 280
Potamonautes perlatus 268
Potamophylax cingulatus 306
Pricea 58
Primates 466
Prionace glauca 314, 316, 317
Prionomys 409
Prionomys batesi 410, 414, 416, 417
Pristiophoriformes 319
Proctoeces 58, 63, 69
Proctoeces maculatus 55, 57, 61
Prokoenenia wheeleri 206, 212, 213
Prolecithophora 41, 42, 56
Promacrostomum gieysztori 39
Promesostomidae 41
Prorhynchidae 41
Prorhynchus 42
Proseriata 41, 42
Prosorchis 58, 59, 63
Prosorchis ghanensis 55, 57, 60
Proteidae 336
Proteocephalus 59
Proteocephalus longicollis 89
Protodrilus 129, 130, 138
Protomicrocotyle 58, 73
Protostrongylus rufescens 122
Protozoa 17
Protura 304
Provortex balticus 46
Provorticidae 41, 46
Psammechinus miliaris 480
Pseudanthobothrium 59
Pseudanthobothrium hanseni 89
Pseudocentrotus depressus 16
Pseudodactylogyrus 58, 73
Pseudodiplorchis 58, 73
Pseudograffilla arenicola 46
Pseudomazocraes 58
Pseudomonas aeruginosa 470
Pseudopythina 163
Pseudopythina rugifera 158
Pseudostomella etrusca 106, 108, 110, 112, 113, 116
Pseudoterranova decipiens 122
Psocoptera 304
Psudocentrotus depressus 17
Pterastericola 46, 75
Pterastericola asamushii 48
Pterastericola astropectinis 46
Pterastericolidae 41, 46
Pterinotrema 58
Pteriomorphia 180
Pteropodidae 422
Pteropodinae 422
Pteropus 424
Pteropus dasymallus 422, 424, 425, 426
Pungitius pungitius 464
Pycnogonum littorale 204
Pycnoporus 58
Pygopodidae 370
Pygopus lepidopus 372
Pyragraphorus 58
Python sebae 372
Pyxicephalus adspersus 336, 339

Q

Quinqueserialis 58
Quiscalus quiscula 438, 440, 441

Source : MNHN, Paris

R

Raillietina (Raillietina) tunetensis 88, 89, 90
Raillietina 59, 89
Raja eglanteria 314, 316, 317, 319
Raja miraletus 57
Raja rhina 507
Rajidae 316
Rajiformes 316
Ramphomyia 306
Ramphotyphlops 385, 386, 387, 390, 393, 394, 395
Ramphotyphlops australis 386, 387, 388, 390, 392, 393, 395
Ramphotyphlops endoterus 386, 387, 388, 390, 392, 393, 395
Ramphotyphlops waitii 386, 387, 388, 390, 392, 393, 394, 395
Rana 57, 98, 327, 328, 448, 465, 466
Rana catesbeiana 455
Rana clamitans 322
Rana dybowskii 322, 324, 327, 328
Rana fuscigula 337, 338
Rana nigromaculata 322, 324, 327, 328
Rana pipiens 322
Rana ridibunda 464
Rana rugosa 322, 324, 328
Ranidae 336
Ranina 277
Ranina ranina 268, 273, 274, 276
Raninoidea 276
Raninoides 268, 273, 276, 277
Raphidioptera 304
Rattus norvegicus 539
Reduviidae 285
Reptilia 359
Retinometra 59
Retinometra serrata 89, 90
Retronectes atypica 38
Retronectes sterreri 38
Rhabditis pellio 122
Rhabdocoela 43
Rhacophoridae 336
Rhacophorus 328, 340
Rhacophorus arboreus 322, 337
Rhacophorus schlegelii 322
Rhea 348
Rhigonema madecassum 122
Rhinobatidae 316
Rhinobatiformes 316
Rhinobatos schlegelii 314, 315, 316, 317
Rhinochimaera pacifica 316, 317
Rhinochimaeridae 316
Rhinolophidae 422
Rhinolophinae 422
Rhinolophus 424, 427
Rhinolophus cornutus 422, 424, 425
Rhinolophus ferrumequinum 422, 424, 425, 427
Rhinolophus imaizumii 422, 425
Rhinolophus luctus 422, 425
Rhinolophus megaphylla 344, 347, 348
Rhinolophus monoceros 422, 425
Rhizodrilus 192, 198
Rhizodrilus russus 192
Rhyacodrilinae 190, 192
Rhyacodrilus 192, 197
Rhyacodrilus arthingtonae 192
Rhyacophila (Hyporhyacophila) tristis 306
Rhyacophila (Pararhyacophila) italica 306
Rhyacophila (Rhyacophila) dorsalis 306
Rhyacophila (Rhyacophila) foliacea 306
Rhyacophilidae 306
Ruditapes decussatus 158
Rugogaster 58, 71, 73

S

Saccharomyces cerevisiae 526
Saccostomus 409, 410, 418
Saccostomus campestris 410, 414, 417
Saguinus imperator 539, 540
Salamandridae 336
Saldula saltatoria 286
Salmo irideus 509
Salmoninae 466
Sandonella 59
Sandonella sandoni 89, 90
Sanguinicolidae 63
Sauria 378
Scatophaga 304, 306
Scatophagidae 306
Schistosoma 55, 56, 57, 58, 62, 70, 71, 74
Schistosoma bovis 57
Schistosoma curassoni 57
Schistosomatium 71
Schizomus 210, 213, 216, 220
Schizomus palaciosi 206, 208, 210, 213
Schizorhynchia 41, 44
Sciara 306
Sciaridae 306
Scincidae 360
Scincomorpha 378
Scintilla 155, 156, 158, 160, 163
Scissurellidae 169, 172
Scissurelloidea 169
Scrobicularia 162, 163
Scrobicularia plana 158, 163, 180, 182
Scrobiculariidae 160
Scutariellidae 41, 47
Scutus 172
Scutus antipodes 169, 172
Scutus sinensis 169
Scutus unguis 169
Scyliorhinidae 316, 466
Scyliorhinus 464
Scyliorhinus canicula 464, 507, 508
Sea cucumber 493, 494
Segestria senoculata 208, 210, 214
Sejus togatus 218
Semnodactylus wealii 336, 337, 338
Sepia officinalis 466
Sergestes arcticus 232, 233, 234, 236, 237

Source : MNHN, Paris

Sergestidae 232, 236
Sericostoma italicum 306
Sericostoma pedemontanum 306
Sericostomatidae 306
Seritia stichopi 46
Serpentes 372, 380
Sicyonia 233, 234
Sicyonia brevirostris 232, 234
Sicyonia carinata 232, 234
Sicyonia ingentis 232, 234, 249
Sicyonidae 232, 234
Silo mediterraneus saturniae 306
Silurana 463, 466, 467
Silurana epitropicalis 466, 467
Sinezona 169, 172
Siphonaptera 304
Siphonops annulatus 336
Siro 208, 210, 211, 212, 224
Siro duricorius 205, 206, 207, 214
Siro rubens 206
Skeneidae 169, 173
Smithsonidrilus 190, 194, 198
Smithsonidrilus hummelincki 189, 190, 191, 192, 195
Solen capensis 158
Solen cylindraceus 158
Solenocera membranacea 232, 233, 234, 236, 239
Solenoceridae 232, 236
Solenoidea 160
Solenopharyngidae 41
Sparidae 466
Sparus aurata 57
Sphaerechinus granulosus 480
Sphaerodactylus cinereus 370
Sphaerolaimus hirsutus 122, 124
Sphenodon 346, 347, 348, 349, 350, 352, 355, 370, 376, 377
Sphenodon punctatus 344, 376
Sphenodontida 355
Sphyranura 58
Sphyrna lewini 314, 315, 316, 317
Sphyrnidae 316
Spirorchis 55, 57, 58, 62, 63
Spisula 453
Spisula solidissima 158, 180, 182, 452, 454, 458, 464
Spisula trigonella 157, 160
Squalidae 316
Squaliformes 316, 319
Squalus 318
Squalus acanthias 466
Squalus brevirostris 316, 317
Squalus japonicus 316, 317
Squamata 356, 359, 376, 385, 466
Squatina japonica 313, 314, 316, 317
Squatinidae 316
Squatiniformes 316, 319
Stactobia caspersi 306
Stallion 520
Steatomys 409
Steatomys parvus 410, 414, 416, 417
Stegonotus cucullatus 372, 374
Stenodactylus selvini 367
Stenophylax permistus 306
Stenoplax conspicua 132, 134, 137
Stenopus hispidus 236, 237, 239
Stilesia 59
Stilesia globipunctata 89, 90
Stimdromia (=Petalomera) lateralis 268, 270, 272
Stimdromia 271, 272, 274, 276
Stophilus multistriatus 299
Stophilus oryzae 299
Strepsiptera 291, 304
Strongylocentrotus intermedius 484
Strongylocentrotus nudus 480
Strongylocentrotus purpuratus 476, 477, 478, 480, 484
Strongylopus 339
Strongylopus grayii 336, 339
Struthio camelus 346
Sturnidae 440
Sturnus vulgaris 438, 440
Styela 465, 502, 503, 504, 512
Styela montereyensis 502, 503
Styela plicata 451, 502, 503, 504
Styelidae 466
Stylops 292, 295
Sus scrofa 539
Sympagurus 254, 256, 260
Symsagittifera 97
Symsagittifera schultzei 98, 100
Syndesmis echinorum 46
Syndisyrinx punicea 46, 48

T

Tachycineta thalassina 439, 440
Tachyglossus aculeatus 539
Tachypleus 204, 210, 216
Tachypleus gigas 205, 208, 216
Tachypleus tridentatus 216
Taenia 59
Taenia hydatigena 89
Talabrica aurora 158
Tapes 20
Tapes japonica 16, 17
Tapes rhomboides 158
Tarantula 213
Tarantula marginemaculata 213
Tarentola mauritanica 370
Tarentola mauritanica mauritanica 370
Taricha granulosa 344, 352, 354
Tarnania 303
Tarsipes rostratus 397, 398, 405, 406
Tatera 409
Tatera leucogaster 410, 412, 413, 417
Tectura scutum 170
Tectus pyramis 169
Teiidae 364
Tellina 160, 162
Tellina rostrata 156, 157, 160, 163
Tellinidae 160
Tellinoidea 160
Telmatobius 321, 327, 328

Source : MNHN, Paris

Telmatobius hauthali 322
Temnocephala 46
Temnocephala dendyi 46
Temnocephala minor 47
Temnocephalida 41, 46
Temnocephalidae 41, 46
Tephritidae 306
Terricola 41
Tetrabothrius 59
Tetragnatha 207, 208, 216
Tetragnatha montana 211
Tetrahymena 15, 16, 17, 18, 20
Tetrahymena pyriformis 16, 17
Tetrahymena thermophila 470
Tetranchyroderma 106, 108, 110
Tetranchyroderma papii 106, 110
Tetranychus urticae 206, 210, 223
Tetraonchoides 58
Tetraonchus 58
Tetrapods 343, 354, 355
Teuthodea 466
Thalassina 258, 260
Thalassina squamifera 252, 254
Thalassinidae 254
Thalassinidea 252, 253, 254
Thalassinoidea 254
Thalassodrilides 198, 199
Thalassodrilides ineri 192
Thalassodrilus 197, 198, 199
Thalassodrilus prostatus 192
Thaumastoderma 108, 116
Thaumastodermatidae 106, 110
Thelastoma periplaneticola 464
Thoracotremata 277
Thraupidae 439
Thunnus thynnus 509
Thyone 25
Thyone briareus 484
Thysaniezia 59
Thysaniezia ovilla 88, 89, 90
Thysanoptera 304
Tiliqua 362, 370, 379, 380
Tiliqua scincoides scincoides 360, 368, 374, 376
Tipula 304, 306
Tipulidae 306
Tivela polita 158
Tomopterna delalandii 336
Tonicella lineata 132, 134, 137
Tonicella marmorea 132
Tonicellidae 132, 146
Torpedinidae 316
Torpediniformes 316
Torpedo tokionis 314, 316, 317
Trachypeneus similis 232, 233
Trapezium sublaevigatum 157, 160
Trepaxonemata 39, 40, 41, 56, 102
Triakidae 316
Triatoma infestans 285, 286, 288
Trichinella nativa 122
Trichinella pseudospiralis 122
Trichinella spiralis 122, 124
Trichocera hiemalis 306
Trichoceridae 306
Trichoptera 301, 303, 304, 308
Trichuris muris 122
Tricladida 41, 43
Tricolia capensis 169
Tridacna maxima 157
Tridacnidae 466
Trigla lucerna 509, 510
Trigonostomidae 41
Trilocularia 59
Trilocularia acanthiaevulgaris 89
Triturus viridescens 336
Trizopagurus strigimanus 254
Trochidae 169, 172,466
Trochoidea 169
Trochopus 58
Trochus nigropunctatus 169
Troglocaridicola 47
Tropiduridae 367
Tropidurus torquatus 367
Tubifex 196, 198, 199
Tubifex tubifex 192
Tubificinae 191, 192
Tubificidae 198
Tubificoides 190, 196, 198, 199
Tubificoides amplivasatus 189, 190, 191, 192
Tubiluchus corallicola 116
Tunicates 502
Turbanella 116
Turbanella ambronensis 106, 108, 112, 114
Turbanella cornuta 106, 108
Turbanellidae 106, 112
Turbinidae 169, 172
Turbo cidaris natalensis 169
Turbo coronatus 169
Turbo sarmaticus 169
Turdus migratorius 439
Turidae 439
Tylenchulus semipenetrans 122
Tymolus 268, 273, 277
Typhlonectes natans 335, 336
Typhlonectidae 336
Typhlopidae 385
Typhloplana virida 43
Typhloplanida 41, 43, 74
Typhloplanidae 41
Tyrannidae 439, 440
Tyrannus verticalis 439
Tyrophagus putrescentiae 223

U

Uca 280
Uca dussumieri 268, 278
Udonella 75
Udonellidae 74
Umagillidae 41, 46
Upogebia pusilla 253
Upogebiidae 253

Source : MNHN, Paris

Uraeotyphlus narayani 336
Uranomys 409, 410, 417, 418
Urastoma 41, 42, 48, 49
Urastoma cyprinae 42
Urodela 336
Urodeles 337
Urolophidae 316
Urolophus aurantiacus 316, 317
Uromastyx philbyi 367
Uroptychus 254, 258
Ursus americanus 539

V

Varanidae 368
Varanus 359, 368, 379, 380
Varanus gouldii flavirufus 347, 367, 368, 369, 370, 376, 380
Varroa jacobsoni 214, 223
Veneroida 180
Veneroidea 158, 160
Venerupis aurea 158
Venerupis corrugata 158
Verutus volvingentis 122
Vespertilio 421, 424, 427
Vespertilio superans 422
Vespertilionidae 422
Vespertilioninae 422
Vetigastropoda 171, 174
Vipera aspis aspis 372
Vireo olivaceus 439
Vireonidae 439

W

Wormaldia copiosa 306, 308
Wormaldia occipitalis 306, 308

X

Xeinostoma 277
Xeinostoma richeri 268, 273
Xenopus 327, 337, 339, 340, 341, 463, 466, 467
Xenopus laevis 322, 334, 337, 338, 455, 467
Xenopus tropicalis 466, 467
Xenos 294
Xenos moutoni 291, 292, 294
Xenos vesparum 291, 292, 294, 295
Xenotrichula 108
Xenotrichula intermedia 106, 112
Xenotrichula punctata 106, 108, 112
Xenotrichulidae 106, 112
Xiphinema diversicaudatum 122
Xiphinema theresiae 122
Xylopagurus 254, 256, 260

Z

Zalipais laseroni 169, 173, 175
Zygentoma 304

Source : MNHN, Paris

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'Chauvinist genes' of male germ cells: gene expression during mouse spermatogenesis. *E. M. Eddy*

A model for understanding gene regulation during spermatogenesis: the mouse testis *Pdha-2* promoter. *Rocco C. Iannello, Julia Young, Sony Sumarsono, Martin Tymms and Ismail Kola*

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Spermatology for understanding, managing and conserving rare species. *David E. Wildt, Budhan Pukazhenthi, Janine Brown, Steven Monfort, JoGayle Howard and Terri Roth*

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Micro-assisted fertilization. *Dianna Payne*

The use of epididymal sperm for assisted reproduction in men with acquired, irreparable obstructive azoospermia. *P. Patrizio , T. Ord, J. P. Balmaceda and R. H. Asch*

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